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Induction of Murine Invariant Chain Gene Expression by Interferon-γ: Definition of an Essential Cis-Acting Element

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Induction of Murine Invariant Chain Gene Expression by Interferon-γ: Definition of an Essential Cis-Acting Element

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ABSTRACT

Interferons are protein factors which are not only involved in the defence against virus infection but play a key role in the regulation of cell growth and differentiation. Many genes have been identified which are specifically induced by interferons and are possible mediators of this complex biological action of interferons. I have analysed the regulation of one of the proteins induced by interferon-γ, the invariant chain, and I have delimited the cis-acting element of the murine invariant chain gene which confers interferon-γ responsiveness in human fibroblasts.

The invariant chain is associated with class II proteins of the major histocompatibility complex and they are all coordinately expressed in cells of lymphoid origin.Their expression can be induced in non-lymphoid cells by treatment with interferon-γ. Experiments described in this thesis show that both the endogenous human gene and the murine gene stably transfected as a cosmid clone are activated by interferon-γ on the transcriptional level in human fibroblasts. This activation requires protein synthesis. Sequence and S1 protection analysis revealed some of the features of the murine invariant chain gene: i) a major start of transcription 29bp 3′ of a TATA box, ii) hetero-geneity in the 5′ untranslated region between different murine strains, iii) a 16 times repeated codon in this region and iv) in contrast to the human gene only one ATG initiator codon.

To identify the regulatory elements of the murine invariant chain promoter responding to interferon-γ, the 5′ flanking region including its cap site and 85bp of transcribed sequences have been cloned in front of the bacterial chloramphenicol acetyltransferase gene. This construct and various 5′ and 3′ deletion mutants of the 5′ flanking region were tested in transient transfection assays and by S1 mapping of RNA from stable transfectants for interferon-γ regulation. DNA sequences between positions -259 and -215 were sufficient to obtain the interferon-γ responsiveness of the complete promoter (7-12-fold). Upon further deletion, induction efficiency dropped to 3-fold (-254 to -215) while constructs not containing this element, or only parts of it were unresponsive to interferon-γ.

Cotransfection of an interferon-γ inducible construct with a 350 molar excess of this interferon-γ response element blocked induction by interferon-γ, suggesting positive regulation by a specific transcription factor. A protein binding to the interferon-γ response element is detectable in an electrophoresis mobility shift assay. It is active only in extracts from interferon-γ treated cells.

ZUSAMMENFASSUNG

Induktion der Expression des Maus "Invariante Kette" Gens durch Interferon-γ : Definition eines essentialen cis-wirkenden Elements.

Interferone sind Proteinfaktoren, die nicht nur an der Abwehr gegen Virus-Infektionen beteiligt sind, sondern auch eine Schlüsselrolle bei der Regulation von Zellwachstum und Differenzierung spielen. Es sind viele Gene identifiziert worden, die spezifisch durch Interferone induziert werden und mögliche Vermittler dieser komplexen biologischen Wirkung von Interferonen sind. Ich habe die Regulation eines der durch Interferon-γ induzierten Proteine, der "Invarianten Kette", untersucht und ich habe das cis-wirkenden Element des Maus "Invariante Kette" Gens eingegrenzt, das die Interferon-γ Antwort in menschlichen Fibroblasten vermittelt.

Die "Invariante Kette" ist mit den Klasse II Proteinen des "Haupthistokompatibilitäts-komplexes" assoziert und wird mit diesem zusammen in Zellen lymphoiden Ursprungs exprimiert. In nicht-lymphoiden Zellen kann ihre Expression durch Behandlung mit Interferon-γ induziert werden. Die vorliegenden Experimente zeigen, daß sowohl das endogene menschliche Gen als auch das stabil in menschlichen Fibroblasten transfizierte Maus Gen durch Interferon-γ auf der Ebene der Transkription aktiviert werden. Für diese Aktivierung ist Proteinsynthese nötig. Durch Sequenzierung und S1 Kartierung des Maus "Invariante Ketten" Gens konnten einige Strukturmerkmale des Gens aufgezeigt werden: i) ein Haupt-Start der Transkription 29bp 3´ von einer TATA-Box, ii) Unterschiede in der 5´ nicht-translatierten Region zwischen verschiedenen Mäuse Stämmen, iii) ein 16 mal wiederholtes Basentriplet in dieser Region und iv) im Unterschied zu dem menschlichen Gen nur ein ATG Start-Kodon.

Um die regulatorischen Elemente des Maus "Invariante Kette" Promoters für die Interferon-γ Induktion zu identifizieren, wurde die 5´ flankierende Region einschließlich "cap site" und 85bp transkribierter Region vor das bakterielle Chloramphenicol-Acetyltransferase Gen kloniert. Dieses Konstrukt und verschiedene 5´ und 3´ Deletionsmutanten der 5´ flankierenden Region wurden in transienten Transfektions-Analysen und durch S1 Kartierung von RNA stabil transfizierter Klone auf Regulation durch Interferon-γ untersucht. DNA Sequenzen zwischen Position -259 und -215 waren ausreichend, um die Interferon-γ Antwort des kompletten Promoters (7-12 fach) zu errreichen. Bei weiterer Deletion ging die Induktion auf 3 fach zurück (-254 bis -215) und Konstrukte, die dieses Element nicht enthielten, oder nur Teile davon, reagierten nicht mehr auf Interferon-γ.

Durch Kotransfektion eines Interferon-γ induzierbaren Konstrukts mit einem 350 fachen molaren Überschuß dieses auf Interferon-γ reagierenden Elements wurde die Interferon-γ

Induktion blockiert, woraus man schließen kann, daß die Induktion durch einen positiv regulatorisch wirkenden Transkriptionsfaktor vermittelt wird. Das auf Interferon-γ reagierende Element bindet ein Protein, wie man mit einer nativen Gelelektrophorese zeigen kann. Das Protein ist nur in Extrakten aus Interferon-γ behandelten Zellen nachweisbar.

CONTENTS

ABBREVIATIONS

approx.	Approximately
APS	Ammonium peroxydisulphate
ATP	Adenosine 5′-triphosphate
bp	Base pairs
BSA	Bovine serum albumin
Bq	Bequerel
CAT	Chloramphenicol acetyltransferase
cos	Cosmid
cpm	Counts per minute
CTP	Cytidine 5′-triphosphate
dATP	Deoxyadenosine 5′-triphosphate
dCTP	Deoxycytidine 5′-triphosphate
ddNTP	Dideoxynucleoside 5′-triphosphates
DEAE-dextran	Diethylaminoethyl-dextran
dGTP	Deoxyguanosine 5′-triphosphate
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DTT	Dithiothreitol
dTTP	Deoxythymidine 5′-triphosphate
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycol-bis-(β-aminoethylether)-N,N,N′,N′-tetraacetic acid
FBS	Foetal bovine serum
Fig.	Figure
g	Gravity
GTP	Guanosine 5′-triphosphate
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid
HBS	High salt buffer
IFN	Interferon
IFN-γ	Interferon-gamma
IN	Invariant chain
IRE	Interferon-gamma response element

kb	Kilobase pairs
kd	Kilodalton
LB	Luria broth
LTR	Long terminal repeat
MCS	Multiple cloning site
MES	2-(N-morpholino)ethanesulphonic acid
MHC	Major histocompatibility complex
min	Minute(s)
MMTV	Mouse mammary tumour virus
M-MLV	Mouse maloney leukaemia virus
mRNA	Messenger ribonucleic acid
PBS	Phosphate buffered saline
PIPES	Piperazine-N,N´-bis(2-ethanesulphonic acid)
PMSF	Phenylmethylsulphonylfluoride
Poly (dIdC)	Polydeoxyinosinic-polydeoxycytidylic acid
RNA	Ribonucleic acid
RNase	Ribonuclease
RNasin	RNase inhibitor
rpm	Rotations per minute
RT	Room temperature
SDS	Sodium dodecyl sulphate
SSB	Sanger sequencing buffer
SSC	Standard saline citrate
SV40	Simian virus 40
TBS	Tris buffered saline
TCA	Trichloroacetic acid
TEMED	N,N,N´,N´-Tetramethylethylenediamine
TFB	Transformation buffer
tk	Thymidine kinase
TLC	Thin layer chromatography
Tris	Tris-(hydroxymethyl)-aminomethane
tRNA	Transfer ribonucleic acid
TTP	Thymidine 5´-triphosphate
U	Unit of enzyme or IFN-γ

INTRODUCTION

Interferons are members of a group of signalling molecules known as lymphokines that are involved in the communication between cells (Hamblin, 1988). Lymphokine mediated events occur during the initiation and effector stages of immune responses and the development of haemopoetic cells. Thus interferons (IFNs) are part of the natural defence system of the body. Initially detected because of their antiviral effects (Isaacs and Lindenmann, 1957; Wheelock, 1965), IFNs are now known to be a group of glycoproteins which exert a multitude of effects on cells both *in vitro* and *in vivo*. These include anti-proliferative, anti-tumour and immunoregulatory activities (reviewed by Pestka et al., 1987).

A large number of different IFNs have been characterized, some of which are encoded by members of a multigene family, while others are encoded by individual genes (Pestka et al., 1987). They can be divided into three subtypes IFNs α, β and γ on the basis of their antigenicity. Both IFN-α and IFN-β have collectively been grouped as type I IFNs, whereas IFN-γ belongs to a distinct group (see Table 1 for a comparison of the different types of IFNs).

Type I IFNs can be induced by viruses, bacteria and by different polymers such as double stranded polynucleotides *in vivo* in leukocytes, fibroblasts and T cells (Kirchner, 1984), whereas IFN-γ is produced solely by activated T lymphocytes (Landolfo et al., 1982). Induction of IFNs results in the stimulation of natural killer cells and macrophages as part of the immune response to these agents. The chemical, 10-carboxymethyl-9-acridanone (CMA), is an effective inducer of IFNs in murine leukocytes, especially in bone marrow derived macrophages. Induction of IFNs by CMA is effective in protecting mice against lethal injections with several RNA and DNA viruses (Storch and Kirchner, 1982). IFNs have also anti-proliferative and anti-tumour effects (Kirchner, 1984; Basham et al., 1986; Pestka et al., 1987). For example, treatment of a breast carcinoma cell line with IFN-α has antiproliferative effects and enhances tumour and HLA

Table A: Comparison of type I and type II interferons

	Type I	Type II
subgroup	α, β	γ
acid stable	yes	no
size	166 amino acids	146 amino acids
no. of genes	α, at least 15 genes β, 2 genes	γ, 1 gene
introns	α, β–1 no; β–2, yes	yes (three)
producer cells	leukocytes fibroblasts	T cells
receptor	α/β receptor	γ receptor

Data compiled from Pestka et al. (1987)

antigen expression (Greiner et al., 1986). Exposure of murine fibroblasts to platelet growth-derived factor induces IFN-β, indicating the function of IFN in a feedback loop control of cell growth (Zullo et al., 1985).

IFN-γ shares all the properties of the classical type I IFNs, despite difference in structure and use of different receptors (see below). It also has additional immunomodulatory effects. In addition to antiviral properties shared with IFNs α and β, IFN-γ is important in macrophage activation and in stimulating macrophage phagocytic and tumorcidal activities (Adolf, 1985). It also increases the resistance of fibroblast and endothelial cells to many intracellular parasites, stimulates B cells and induces cell surface proteins such as class II antigens of the major histocompatibility complex (see below) central to immune cell regulation (Adolf, 1985). Acting synergistically with lymphotoxin, another lymphokine, IFN-γ has greater cytostatic and cytotoxic effects than IFNs α and β (Stone-Wolff et al., 1984).

The effects of IFNs on their target cells are initiated by their binding to specific cell receptors (Aguet, 1980). A factor contributing to the uniqueness of IFN-γ is that it does not share or compete for binding to the receptors for type I IFNs

(Aguet et al., 1982; Branca and Baglioni, 1981). Instead it binds to distinct receptors (Littman et al., 1985; Aiyer et al., 1986). In the human, receptors encoded by chromosome 21 alone are sufficient for a response to IFNs α and β whereas a receptor encoded by chromosomes 6 and a factor encoded by chromosome 21 are necessary for a response to IFN-γ (Jung et al., 1987).

Binding of IFNs to their receptors results in the synthesis of specific polypeptides: as revealed by 2-dimensional polyacrylamide gel electrophoresis, treatment of fibroblasts with IFNs α and β results in the detection of at least 12 new polypeptides (Weil et al., 1983). IFN-γ induces the synthesis of a set of polypeptides some of which are in common with those produced in response to type I IFNs while others are unique to IFN-γ (Weil et al., 1983). This correlates with the additional functions of IFN-γ in addition to those in common with type I IFNs. Common proteins may represent products of converging transduction pathways in the cellular response to both classes of IFN. An example of one polypeptide whose expression is induced by all IFNs (although less efficiently by IFN-γ than by IFNs α and β) is 2′, 5′-oligoadenylate synthetase. This protein is involved in the antiviral effects of the IFNs (Lengyel, 1982). All IFNs also induce the expression of class I antigens of the major histocompatibility complex (MHC) but only IFN-γ significantly induces the expression of the class II antigens and their associated invariant chain (Sugita et al., 1987; Basham and Merigan, 1983).
In several cases, exposure to IFN has been shown to cause an increase in mRNA synthesis. For example, IFN-γ treatment of dermal fibroblasts results in the increase of class II antigens and invariant chain mRNA levels (Collins et al., 1984) and all IFNs induce the transcription of the 2′, 5′-oligoadenylate synthetase gene (Friedman et al., 1984; Faltynek et al., 1985). However the mechanism by which the signal generated by IFN is transferred from the cell surface to the nucleus is still unknown.

Gene activity is regulated primarily at the level of transcription (Serfling et al., 1985). In addition, regulation can also occur at the post-transcriptional level (Shapiro and Brock, 1985). In transcriptional activation, conserved DNA

elements, known as promoters (or cis-acting elements) govern the regulation. In higher eukaryotes, an AT-rich sequence about 30 base pairs (bp) upstream of the RNA initiation site (TATA box) is an indispensible promoter component. An additional conserved sequence, the CAAT box, is located upstream of many eukaryotic genes (reviewed in Breathnach and Chambon, 1981). Other promoter elements known as enhancers are responsible 1) for the general stimulation of transcription, 2) tissue specific gene expression and 3) the induction (or repression) of transcription by the action of specific agents. There is considerable variation in the location and sequence of many cis-acting elements (reviewed by Serfling et al., 1985). The activity of cis-acting elements is dependent on the presence of cellular trans-acting factors, proteins which bind to them (Schöler and Gruss, 1984). The interaction between these cis- and trans-acting elements which determine the rate of transcription, constitutes the ultimate step in signal transduction.

Peliminary investigations have shown that the mechanisms of gene activation by IFNs are different for type I and type II IFNs: IFN-γ requires protein synthesis for the activation of 2′, 5′-oligoadenylate gene expression whereas type I IFNs do not (Faltynek et al.,1985; Friedman et al., 1984). Since the start of this work, further studies on the regulation of gene expression by type I IFNs have led to the definition of the cis-acting sequences which confer IFN α/β inducibility to genes (Friedman and Stark, 1985; Levy et al., 1988; Sugita et al., 1987; Cohen et al., 1988; Porter et al., 1988). It has also been shown that these cis-acting sequences have enhancer properties and that proteins bind to them (Cohen et al., 1988; Porter et al., 1988).

The aim of this thesis was to study the mechanism by which IFN-γ activates gene expression since nothing was known (at the start of this work) about its mechanism of gene activation. I chose to study the induction of the class II associated invariant chain as an example of an IFN-γ regulated gene, with the ultimate aim of defining the cis-acting sequences which confer IFN-γ inducibility to the gene.

Class II antigens of the major histocompatibility complex (MHC) are expressed on B cells, some T cells and on antigen presenting cells such as macrophages (Cowing et al., 1978). The class II molecules are composed of two polymorphic chains, α (33-35kilodalton (kd)), and β (27-29kd), both encoded in the MHC (mouse, I-A and I-E regions; man, HLA-D region; Steinmetz et al., 1982; Kaufmann et al., 1984). The α and β chains of class II antigens are assembled and glycosylated in the endoplasmic reticulum, transferred to the golgi apparatus for further glycosylation before being transported to the cell surface where they are membrane associated. Class II antigens are important in the presentation of foreign antigen to T cells (Benacerraf, 1981). T cells recognise foreign antigenic determinants on the surface of an antigen presenting cell only in conjunction with self class II antigens.

Associated intracellularly with the class II antigens is a third chain (Marks and Cresswell, 1986), γ-chain or now called invariant chain (IN). It is so named because it exhibits no variation in isoelectric point as identified by 2-dimensional polyacrylamide gel electrophoresis. It was first detected in immunoprecipitates of the murine class II antigens because it coprecipitates with antibodies to the I-A and I-E regions of the MHC (Jones et al., 1978). The IN has a molecular weight of 31kd in mice and 33kd in humans with several minor forms ranging from 25kd to 41kd (Zecher et al., 1984). The IN, like the class II polypeptides is glycosylated (Machamer and Cresswell, 1982), but its membrane orientation is the opposite to the class II antigens with the amino terminal end located in the cytoplasm (Lipp and Dobberstein, 1986). Characteristic of this type of membrane protein, it has no signal sequence (Lipp and Dobberstein, 1986). The IN is encoded by a single gene outside the MHC (Day and Jones, (1983), on chromosome 5 in humans (Claesson-Welsh et al., 1984) and on chromosome 18 of the mouse (Yamamoto et al., 1985a). The different forms of the IN within a species are due to alternate splicing of RNA and, in the human, due also to alternate starts of translation (Yamamoto et al., 1985b; Strubin et al., 1986a, 1986b; O´ Sullivan et al., 1987).
The function of the IN is still unknown. Since it is present in cells expressing class II antigens and because of its physical association with class II antigens,

functions in relation to this association have been postulated. It has been suggested that the IN plays a role in the assembly or the intracellular transport of the α and β chains of the class II antigens (Claesson and Peterson, 1983; Claesson-Welsh and Peterson, 1985). On the other hand, there is evidence that the association between the IN and class II antigens is transient and that the IN protein is processed to a complex oligosaccharide form which reaches the cell surface independently of class II molecules (Holt et al., 1985; Sant et al., 1985). It has also been shown that class II antigens are expressed on the cell surface in cells lacking detectable IN (Miller and Germain, 1986; Schneider et al., 1987; Sekaly et al., 1986, 1988). It would thus seem that the IN is neither involved in the assembly nor the intracellular transport of the class II antigens.

Since IN itself seems to be expressed on the cell surface (Koch et al., 1982; Claesson and Peterson, 1983; Holt et al., 1985; Sant et al., 1985), it possibly plays some role in the function of the class II antigens. Sekaly et al. (1988) have reported that antigen presentation to HLA class II restricted T cell clones can occur in the absence of the IN *in vitro,* but on the other hand, Stockinger et al. (1988) provide strong evidence to demonstrate that the IN is crucial in the presentation by class II antigens of intact foreign antigens to T cells. They suggest that the IN is essential for antigen processing, which involves the internalization and degradation of intact antigen before it can be presented to T cells.

Not only is the IN physically associated with the class II antigens, it is also coordinately regulated with them (Rahmsdorf et al, 1983; Collins et al., 1984; Koch, N. et al., 1984; de Préval et al., 1985; Paulnock-King et al., 1985). As already stated, IFN-γ induces the expression of the class II antigens and their associated IN. This induction occurs not just in cells of lymphoid origin but in other cells, such as fibroblasts (Collins et al., 1984; Rahmsdorf et al., 1986); macrophages (Paulnock-King et al., 1985); gliomas (Takiguchi et al., 1985); T-dependent mast cells (Koch et al., 1984) and human endothelial cells (Collins et al., 1984).

To investigate how IFN-γ regulates the synthesis of the murine IN gene the following strategy was used. The gene coding for the murine IN was transfected into fibroblast cells which do not express IN constitutively and was analysed for expression in the absence and presence of IFN-γ. Human fibroblasts were chosen because the transfected murine gene could be distinguished from the endogenous human IN gene by the use of different DNA probes.
First, the requirement for protein synthesis in the activation of the IN gene by IFN-γ was investigated. Then the level at which IFN-γ regulates the expression of the IN gene was analysed, whether this regulation is at the transcriptional or post-transcriptional level. Finally, the cis-acting sequences involved in conferring IFN-γ inducibility to the gene were determined.

Recombinant IFN-γ was used in this analysis because since the cloning and expression of the gene for human IFN-γ in bacteria (Gray et al., 1982), it can be prepared in large quantities and purified to homogeneity in a biologically active form. It was used because effects due to IFN-γ alone can be studied, since preparations of natural IFN-γ often contain contaminating lymphokines which act synergistically to enhance IFN-γ's properties (Gray and Goeddel, 1987). Recombinant IFN-γ has confirmed and extended much of the earlier work in the "classical" areas of IFN research and has even revealed new properties of this lymphokine (Adolf, 1985; Gray and Goeddel, 1987).

In this thesis I have shown that human IFN-γ activates the transcription of the murine IN gene transfected into human fibroblasts. I have characterized the transfected gene and identified its 5′ flanking sequences where regulatory sequences are normally located. By analysis of deletion mutants, I defined the sequences which confer IFN-γ inducibility to the gene. I also demonstrated that IFN-γ activation of IN transcription through the IFN-γ response element is mediated by a positively acting factor. Finally, an IFN-γ inducible protein complex was shown to bind to the IFN-γ response sequences in DNA-protein binding studies.

MATERIALS

1. Chemicals, Reagents and Materials

Acetyl coenzyme A	Pharmacia, Freiburg
Acridine orange	Sigma, München
Acrylamide	Serva, Heidelberg
Actinomycin D	Sigma, München
Agarose type II	Sigma, München
Alkaline phosphatase	Boehringer, Mannheim
Ammonium peroxydisulphate	Bio-Rad, München
Ampicillin	Sigma, München
Bal 31 exonuclease	Gibco-BRL, Karlsruhe
Biogel P60	Bio-Rad, München
BSA	Sigma, München
Caesium chloride	Biomol, Ilvesheim
Creatine phosphokinase	Sigma, München
Cycloheximide	Sigma, München
DE81 DEAE-cellulose paper	Bender and Hobein, Karlsruhe
DEAE-dextran	Sigma, München
Deoxynucleotide triphosphates	Boehringer, Mannheim
Dichlordimethylsilane	Fluka, Neu-Ulm
Dideoxynucleotide triphosphates	Boehringer, Mannheim
Diethylpyrocarbonate	Sigma, München
Dimethylsulphoxide	Fluka, Neu-Ulm
Dithiotreitol	Gibco-BRL, Karlsruhe
DNA polymerase 1 (Klenow fragment)	New England Biolabs, Schwalbach
DNA sequencing kit	New England Nuclear, Dreieich
Ethanol	Roth, Karlsruhe
Ethidium bromide	Sigma, München
Ficoll	Serva, Heidelberg
Foetal bovine serum	Gibco-BRL, Karlsruhe
Formamide	Fluka, Neu-Ulm
Glass plates for electrophoresis	Renner, Darmstadt
Glycerol	BRL Inc., Neu-Isenburg
G418	Gibco-BRL, Karlsruhe
HEPES	Sigma, München
Linker DNAs	New England Biolabs, Schwalbach
Lysozyme	Boehringer, Mannheim
M-MLV reverse transcriptase	Gibco-BRL, Karlsruhe
NACS prepack columns	Gibco-BRL, Karlsruhe
Nick translation kit	Amersham Buchler, Braunschweig
Nitrocellulose filter	Schleicher & Schüll, Dassel
N,N´-Methylene bisacrylamide	Bio-Rad, München
Nonidet P-40	Sigma, München
Nucleoside triphosphates	Boehringer, Mannheim
Oligo$(dT)_{12-18}$- cellulose type VII	Collaborative Research Inc.

Oligonucleotide synthesizer	Pharmacia, Freiburg
Penicillin/Streptomycin	Gibco-BRL, Karlsruhe
Phosphocreatine	Sigma, München
PIPES	Sigma, München
PMSF	Sigma, München
Poly (dIdC) . poly (dIdC)	Pharmacia, Freiburg
Proteinase K	Merck, Darmstadt
Polyvinyl pyrollidone	Sigma, München
Quickszint	Zinsser, Frankfurt
Restriction and modification enzymes	ABL, Basel Boehringer, Mannheim Gibco-BRL, Karlsruhe Promega Biotec, Heidelberg
RNase A	Boehringer, Mannheim
RNasin	Promega Biotec, Heidelberg
Salmon sperm DNA	Sigma, München
SDS	Serva, Heidelberg
Silica gel plates for TLC	Macherey-Nagel, Düren
S1 nuclease	Gibco-BRL, Karlsruhe
SP6 polymerase	New England Biolabs, Schwalbach
TEMED	Bio-Rad, München
tRNA	Boehringer, Mannheim
T4 DNA ligase	Boehringer, Mannheim
T4 polynucleotide kinase	New England Biolabs, Schwalbach
Tris	Sigma, München
Trypsin	Gibco-BRL, Karlsruhe
Urea	Serva, Heidelberg
Whatman GF/C filters	Bender and Hobein, Karlsruhe
Whatman 3MM paper	Bender and Hobein, Karlsruhe

Chemicals for transformation of bacteria were obtained from Fluka, Neu-Ulm. All other chemicals (p. A. grade) which are not listed above were purchased from Merck, Darmstadt. The solid substances for bacterial culture medium were obtained from Difco laboratories, Detroit, those for eukaryotic cell culture from Gibco-BRL, Karlsruhe. Plastic cell culture flasks and petri dishes were obtained from Greiner, Nürtingen.

2. Radioisotopes

All were obtained from Amersham Buchler, Braunschweig:

D-threo-(dichloroacetyl-1-^{14}C)-Chloramphenicol (7.4MBq/ml, 1.96GBq/mmol)

$\gamma\,^{32}$P ATP (370MBq/ml, >185TBq/mmol)

$\alpha\,^{32}$P dCTP (370MBq/ml, >15TBq/mmol)

$\alpha\,^{35}$S dATP (296MBq/ml, >14.8TBq/mmol)

$\alpha\,^{32}$P UTP (370MBq/ml, >110TBq/mmol)

3. Interferon

Human recombinant interferon-γ (IFN-γ) was a gift from Dr. G. R. Adolf, Ernst-Boehringer-Institut für Arzneimittelforschung, Vienna. It has a specific activity of $1\text{-}3\times10^7$U/mg. IFN-γ is assayed by inhibition of viral replication in tissue culture. In this test, the amount of IFN-γ that in a dilution series causes an inhibition of viral replication by 50% is defined as one International Unit (the use of standards is mandatory).

4. Murine Strains

Balb/c (H-2^d), AKR (H-2^k), C57Bl (H-2^b), one to three months old were purchased from the Zentralinstitut für Versuchstierzucht, Hannover.

5. Bacteria and Eukaryotic cells

Bacteria

E. coli RR1 M15	F^-, hsd S20, ara-14, pro A2, lac Y1, ton A21, sup E44, I^- From U. Rüther, Heidelberg

Eukaryotic cells

GM637	Human skin fibroblasts transformed with simian virus 40. From J.E. Cleaver, University of California, San Francisco
CH1.1	A variant tumor cell line of the murine Ia^+ B cell lymphoma, CH-1, (Lynes et al., 1978; Rahmsdorf et al., 1983; Koch, S, et al., 1984)

6. Plasmids

cos 10.7	Genomic cosmid clone containing the entire murine invariant chain gene. From M. Steinmetz, Basel (Yamamoto et al., 1985b).
α-actin	Partial cDNA clone (containing about 1350bp or 90% of the coding sequences, cloned in the Pst I site of pBR322) of the murine α-actin gene. From M. Buckingham, Paris (Minty et al., 1981).
P900 (murine IN cDNA)	Partial cDNA clone (+350 to poly A site in the Pst I site of pBR322) of the murine invariant chain gene. From B. Dobberstein, Heidelberg (Singer et al., 1984).
human IN	Human IN cDNA, isolated by H. J. Rahmsdorf, it is an internal 150bp Pst I fragment from IN-8 (Rahmsdorf et al., 1986) cloned into pUC9.

7. Culture medium

a. Bacteria culture medium:

Luria-Broth (LB)	1% Bactotryptone 0.5% Yeast extract 0.5% NaCl
solid medium	LB containing 1.5% agar

b. Culture medium for transformation of bacteria:

SOB	2% Bactotryptone 0.5% Yeast extract 10mM NaCl 2.5mM KCl 10mM $MgCl_2$ 10mM $MgSO_4$
SOC	SOB containing 20mM Glucose

c. Selection medium for bacteria:

LB supplemented with either of the following antibiotics :
Ampicillin (100µg/ml) or Tetracycline (30µg/ml)

d. Culture medium for GM637 cells:

Dulbecco's modified essential medium (DMEM)
10% Foetal bovine serum (FBS)
Penicillin (100U/ml)
Streptomycin (100µg/ml)

e. Culture medium for CH1.1 cells:

RPMI 1640
10% FBS
Penicillin (100U/ml)
Streptomycin (100µg/ml)
2mM Glutamine
50µM 2-mercaptoethanol

METHODS

The protocols used were taken from the laboratory manual of Maniatis *et al* (1982) unless otherwise stated.

I. GENERAL METHODS

1. Determination of nucleic acid concentration

The concentration of nucleic acids in H_2O was determined by measuring their optical density (O.D.) at 260 and 280nm. An $O.D._{260}$ = 1 is equivalent to 50µg/ml of double stranded DNA or 40µg/ml RNA or 20µg/ml of oligonucleotides. The $O.D._{280}$ is used as an indication of the purity of the nucleic acid; it should be approximately 50% of the $O.D._{260}$.

2. Purification of nucleic acids

a. Phenol/chloroform extraction

To remove proteins from nucleic acids, a phenol/chloroform extraction was used. An equal volume of phenol (saturated with 10mM Tris-HCl pH 7.5, 100mM NaCl, 5mM EDTA) was added to the nucleic acid and mixed in. The same volume of chloroform/isoamylalcohol (24:1) was added and the solution was mixed again by gentle vortexing. The two phases were separated by brief centrifugation. The upper aqueous phase was extracted again with an equal volume of chloroform/ isoamylalcohol.

b. Ethanol precipitation

To recover nucleic acids from solution, the salt concentration was brought to 0.2M with sodium acetate (pH 4.8), and a 2.5-fold volume of ethanol was added and mixed in. After a 30 min incubation at -20°C, the precipitate was pelleted by centrifugation for 20 min at 9000×g. The pellet was washed with 80% ethanol to remove the salt, centrifuged again and dried under vacuum.

3. Size separation of nucleic acids by electrophoresis

a. Agarose gels

The required amount of agarose (final concentration between 0.8 and 2%) was dissolved by heating in 50ml electrophoresis buffer (TBE: 90mM Tris, 90mM boric acid, 2.5mM EDTA, pH 8.3). Ethidium bromide was added at a concentration of 0.2µg/ml. The molten gel was poured into a horizontal (13.5×8cm) chamber. Combs with the appropriate number of teeth were used to make the loading slots. The gel (when set) was covered with 200ml electrophoresis buffer and run at 100mA at RT for the required time. Samples were loaded onto the gel in loading buffer (10mM EDTA, 10% glycerol, 0.1% SDS, 0.02% bromophenol blue). DNA was visualised by transillumination with long wave (302nm) ultra-violet light. A permanent record of the gel was made by photography using a polaroid land camera.

b. Polyacrylamide gels

6% polyacrylamide gels were made from a stock solution of 29% acrylamide and 1% N,N´-methylene bisacrylamide (w/w), in the same electrophoresis buffer as for agarose gels. Polymerization was achieved by the addition of TEMED to 0.05% and ammonium persulphate to 0.1%. Gels were 0.1cm thick between two glass plates (12×15cm) and were run vertically. Electrophoresis was performed at 20mA until the tracking dye had migrated as far as required. The gels were stained with ethidium bromide (1µg/ml H_2O, 15 min) and DNA was visualised as in 3a.

c. Denaturing polyacrlyamide gels for the analysis and separation of reaction products of DNA sequencing, S1 analysis, primer extension and RNA sequencing.

A denaturing polyacrylamide gel was prepared as follows: 6% acrylamide (see 3b) and 8M urea in TBE (3a) were mixed with TEMED to 0.05% and ammonium persulphate to 0.1% and immediately poured between two glass plates (20×40cm) 0.3mm apart. One glass plate was siliconized beforehand with 3ml 5% dichlorodimethylsilane in CCl_4. Following a pre-run at 30W for 30 min, the denatured samples were loaded onto the gel and electrophoresis took place for the required time at 30W. TBE was used as the electrophoresis buffer. The samples were prepared as follows: the dried reaction products were resuspended in 3µl SSB (SSB:

90% deionised formamide, 20mM EDTA, 0.03% xylene cyanol, 0.03% bromophenol blue). They were denatured by incubation at 85°C for 5 min, directly transferred to ice for 2 min, then loaded immediately onto the gel.

At the end of the electrophoresis, the glass plates were separated, and the gel (still on the unsiliconized plate) was fixed in 10% acetic acid/10% methanol (in H_2O) for 10 min. The gel was then transferred to 3MM Whatman filter paper and dried under vacuum. The separated reaction products were visualised by autoradiography - the gel was exposed to Kodak XAR-5 film with an intensifying screen at -80°C.

II. DNA CLONING TECHNIQUES

1. Restriction endonuclease digestion

One unit of activity of a restriction enzyme is defined as the amount of enzyme required to completely cut 1µg of lambda phage DNA in one hour under optimal conditions. Usually a 2-3 fold excess of enzyme was used to ensure complete digestion. Partial digestion was achieved by incubation of 1µg DNA with 0.5-1U enzyme at RT and aliquots were removed at various time intervals. DNA was digested at a concentration of 1µg/10µl in a "general" buffer (10mM Tris-HCl pH 8.0, 10mM $MgCl_2$, 1mM DTT, 50mM NaCl) at 37°C for most enzymes. For the enzymes Stu I and Mae III, buffers recommended by the suppliers were used. The reaction was stopped by a phenol/chloroform extraction and the DNA was precipitated with ethanol.

2. Dephosphorylation of DNA

a. 5′ overhangs

Restriction enzyme digested DNA was incubated with one unit of alkaline phosphatase (from calf intestine) per 100pmol DNA ends in 50µl 50mM Tris-HCl pH 9.0, 1mM $MgCl_2$, 0.1mM $ZnCl_2$, 1mM spermidine at 37°C. After 30 min, one unit enzyme was added again and incubation was continued for another 30 min.

Phosphatase was inactivated by incubation at 68°C for 10 min. Phenol/chloroform extraction and ethanol precipitation (section I.2) were carried out to recover the DNA for further procedures.

b. 3′ overhangs and blunt ends

This followed basically the same procedure except that incubation was carried out for only 15 min at 37°C followed by 15 min at 56°C. This cycle of incubation was repeated after addition of new enzyme.

3. "Fill-in" of 5′ overhangs

1µg of digested plasmid DNA was incubated with 1U of DNA polymerase I (Klenow fragment) in 10µl 7mM Tris-HCl pH 7.5, 7mM $MgCl_2$, 50mM NaCl, 1mM DTT, 50µM dNTPs for 10 min at RT. Phenol/chloroform extraction and ethanol precipitation were carried out as described in I.2.

4. Removal of 5′ overhangs

1µg of restricted plasmid DNA was incubated with 1U S1 nuclease in 0.3M NaCl, 30mM CH_3COONa, 3mM $ZnSO_4$ at RT for 5 min. The DNA was precipitated with ethanol after phenol/chloroform extraction (See section I.2).

5. Bal 31 digestion of DNA

Bal 31 degrades both the 3′ and 5′ strands of DNA in a progressive manner. Under suitable conditions, a linear DNA molecule can be digested from both ends in a controlled fashion as follows: 25µg of restricted plasmid DNA were incubated in 300µl Bal 31 buffer (12mM $CaCl_2$, 12mM $MgCl_2$, 200mM NaCl, 1mM EDTA, 20mM Tris-HCl pH 8.1) with 5U Bal 31 at 37°C. At intervals of 30 seconds, an aliquot of 75µl was removed into a solution of EGTA (final concentration, 20mM EGTA) to stop the reaction. By varying the time intervals, deletions of different lengths can be made. The shortened DNA was precipitated with ethanol after phenol/chloroform extraction. (See section I.2).

6. Isolation of DNA fragments from gels

a. Elution of DNA from agarose gels (modified from Dretzen et al., 1981)

DNA was separated on an agarose gel and visualised under UV illumination as described in I.3a. A cut was made in the agarose with a scalpel, about 2mm below the fragment to be isolated. A strip of DE81 DEAE-cellulose paper was inserted into the slit and electrophoresis was continued until the DNA fragment had run onto the paper. The latter was removed from the gel and washed briefly with distilled H_2O. DNA was eluted by incubation of the paper (torn into small pieces) in 100-400µl 1.5M NaCl at 65°C for 15 min. The solution was then passed through glass wool in an eppendorf tube to remove the DE81 paper. A hole was made in the bottom of this eppendorf tube so that the fluid could be collected in a second eppendorf tube underneath. The DNA fragment was recovered from the 1.5M NaCl solution by ethanol precipitation.

b. Elution of DNA from polyacrylamide gels

DNA was eluted from polyacrylamide gels by incubation of the relevant gel slice in 0.2M NaCl in TE (10mM Tris-HCl pH 7.2, 1mM EDTA) at 50°C overnight. The DNA was purified over glass wool followed by ethanol precipitation.

7. Ligation of DNA

a. Ligation of restriction fragments

The DNA fragments to be ligated were mixed in equimolar amounts with 4U T4 DNA ligase in a final volume of 20µl of 20mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 250µg/ml BSA and 1mM ATP and incubated at 15°C for 4-16 hours.

b. Ligation of linkers

1µg linkers were phosphorylated with 10U polynucleotide kinase in the presence of 10mM ATP. Incubation took place at 37°C for 30 min in 10µl 70mM Tris-HCl pH 7.6, 10mM $MgCl_2$. 2µl of phosphorylated linkers were added to 1µg purified blunt-end DNA. This was incubated in a final volume of 10µl containing 70mM Tris-HCl pH 7.5, 7mM $MgCl_2$, 0.1mM ATP and 4U T4 DNA ligase at 15°C overnight. Ligase was

inactivated by a 10 min incubation at 65°C. The volume was then increased to 30µl and a restriction enzyme digest was carried out for a minimum of 3 hours with 20-40 U enzyme to trim the linkers. The linker-ligated DNA was separated from excess free linkers by agarose gel electrophoresis.

8. Cloning of oligonucleotides

Oligonucleotides were synthesized on a Gene Assembler from Pharmacia. They were eluted from the support column by overnight incubation at 50°C in 1ml 25% ammonium. 1µl from each of the complementary strands was dried down in a vacuum centrifuge and resuspended in 100µl 6.25mM Tris-HCl pH 8.5, 3mM $MgCl_2$. Equimolar amounts of a 1:50 dilution of the oligonucleotides in the same buffer were heated at 100°C for 2 min and then allowed to cool slowly to RT to anneal the complementary strands. 2µl from this double stranded oligonucleotide was ligated into 100ng vector as described above.

9. Purification of oligonucleotides (for use as primers or in electrophoresis mobility shift assays)

(see I.3c): An 18% denaturing polyacrylamide gel, 1mm thick was used for the separation of full length oligonucleotide synthesis products from shorter incomplete molecules. 50µg of single stranded oligonucleotide was resuspended in in SSB, loaded onto a 2cm wide slot and electrophoresis was carried out at 25W for 3 hours. The gel was stained with ethidium bromide and the bands were visualised as described in I.3a. The band containing the full length product was cut out with a scalpel and the DNA was eluted by incubation of the crushed gel slice in H_2O overnight at 37°C. The DNA was purified over a Nacs Prepac column following the manufacturer's instructions.

10. Transformation of bacteria (modified from Hanahan, 1983)

Preparation of competent *E. coli*: A single colony of *E. coli* RR1 M15 was innoculated into 50ml SOB medium and grown to an $O.D._{600}$ 0.3-0.35. After incubation on ice for 10 min, the bacteria were sedimented by centrifugation at 3600×g for 15 min at 4°C. The bacteria were resuspended in 5ml TFB (100mM KCl,

45mM $MnCl_2$, 10mM $CaCl_2$, 3mM Hexamine $CoCl_3$, 10mM K-MES pH 6.2, 15% glycerol) and incubated on ice for a further 15 min. The cells were centrifuged again and resuspended in 2ml TFB. They were either directly transformed or frozen in liquid nitrogen in aliquots.

Transformation: 5-10µl of ligation mix was added to 150µl of competent cells. After incubation on ice for 30 min, the cells were heat shocked at 42°C for 90 seconds and were returned to ice for 2 min. 2ml SOC medium was added and the bacteria were incubated at 37°C, with shaking for 45 min. The cells were pelleted by a short centrifugation (3 min, 1000×g), resuspended in 0.2ml SOC and plated out onto LB-agar plates supplemented with an appropriate antibiotic which were then incubated overnight at 37°C.

11. Plasmid DNA preparation from bacteria

a. Mini preparation

1.5ml LB medium containing a suitable antibiotic was innoculated with a single colony and incubated overnight at 37°C until the bacteria reached stationary phase. The bacteria were pelleted by centrifugation at 5600×g for 5 min and were resuspended in 100µl solution I (2mg/ml lysozyme, 50mM glucose, 10mM EDTA, 2.5mM Tris-HCl pH 8.0). After 30 min incubation on ice, 200µl solution II (0.2M NaOH, 1%SDS) were added. Following a further 5 min on ice, 150µl solution III (3M sodium acetate) were added and incubation continued for 60 min on ice. After a 5 min centrifugation at 5600×g, 1ml ethanol was added to the supernatant and the DNA was precipitated at -20°C for 30 min. The plasmid DNA was pelleted by centrifugation for 10 min at 5600×g and resuspended in 100µl solution IV (50mM Tris-HCl pH 8.0, 0.1M sodium acetate). It was precipitated again with ethanol at -20°C for 30 min. After centrifugation, the plasmid DNA was dried under vacuum and resuspended in 40µl H_2O and stored at -20°C.

b. Large scale plasmid preparation

200ml LB medium (supplemented with the relevant antibiotic) were innoculated with 0.1ml of a bacteria overnight culture and incubated with aeration at 37°C overnight until the bacteria had reached stationary phase. The bacteria were pelleted

by centrifugation at 3600×g at 4°C for 10 min, resuspended in 10ml solution I (see mini plasmid prep), and incubated on ice for 30 min. 20ml solution II were added and after a further 5 min on ice, 15ml solution III were added. Following an incubation on ice for 60 min, the cell fragments and chromosomal DNA were sedimented by centrifugation at 13200×g for 20 min at 4°C. The plasmid DNA in the supernatant was precipitated by the addition of 100ml ethanol and incubation at -20°C for 30 min. The DNA was pelleted by centrifugation at 8600×g at 4°C for 10 min, was resuspended in 10ml solution IV and precipitated again with ethanol as above. Finally, it was resuspended in 3ml TE (50mM Tris-HCl pH 8.0, 1mM EDTA). Caesium chloride was added to the DNA at a final concentration of 4.2M and ethidium bromide to 0.6mg/ml. This solution was transferred to a Beckman quick-seal tube and centrifuged in a Beckman vertical rotor type 65 for 16 hours at 55000 rpm at 20°C. The resulting caesium chloride gradient separated the plasmid DNA from bacterial RNA and proteins. The band containing supercoiled plasmid DNA was removed using a hypodermic syringe and was centrifuged again under the same conditions for 6 hours. The plasmid containing band was removed again, diluted twofold with H_2O and extracted with water-saturated butanol three times to remove the ethidium bromide and the DNA was precipitated with ethanol at -20°C overnight. After centrifugation, the plasmid DNA was dried under vacuum and resuspended in 500µl H_2O and stored at -20°C.

c. Cosmid DNA preparation

Bacteria were grown on 25×25cm agar plates (to minimise the rearrangement of cosmid DNA) at a density of 25,000 colonies per plate. The bacteria were harvested with a rubber spatula and the plates were washed twice with 10ml LB medium to collect all the bacteria. The bacteria were centrifuged at 3600×g at 4°C for 10 min and cosmid DNA was prepared as described above for plasmid DNA.

12. Radioactive labelling of DNA

a. Nick Translation

500ng DNA was labelled with the nick translation kit from Amersham Buchler, Braunschweig. The DNA was incubated with 10µl solution I (100µM each dATP,

dTTP, dGTP in 10mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 5mM 2-mercaptoethanol) in the presence of 1.85MBq $\alpha^{32}P$ dCTP and 5µl solution II (2.5U DNA polymerase I and 100pg DNase I) in a final volume of 50µl. Incubation took place at 15°C for 3 hours. The reaction was stopped by the addition of 1µl 0.5M EDTA and 1µl 20% SDS. Radioactively labelled DNA was separated from unincorporated nucleotides by gel filtration through Biogel P60 as follows: A pasteur pipette stoppered with glass wool was used as a column into which Biogel P60 (suspended in 50mM NaCl, 0.5mM EDTA) was filled. After loading the sample onto the column, it was eluted with the same buffer and 100µl fractions were collected. The fractions containing the DNA were identified by Cherenkow counting and combined.

b. Multiprime labelling (Feinberg and Vogelstein, 1983)

The multiprime kit from Amersham Buchler, Braunschweig, was used to label 25ng DNA according to the accompanying instructions. The DNA was first denatured in 29µl H_2O by heating to 100°C for 10 min and rapidly cooling on ice. 10µl solution I (dATP, dTTP, dGTP in Tris-HCl pH 7.5, 10mM $MgCl_2$, 5mM 2-mercaptoethanol), 5µl solution II (random hexanucleotides as primers), 1µl solution III (1U/ml DNA polymerase I, Klenow fragment) and 1.85 MBq $\alpha^{32}P$ dCTP (5µl) was added. The reaction took place at RT for 5-16 hours. Radioactively labelled DNA was separated from unincorporated nucleotides by gel filtration through Biogel P60 as described above.

c. 5´ end labelling of restriction fragments

200-600ng dephosphorylated DNA were incubated with 10U polynucleotide kinase and 3.7MBq $\gamma^{32}P$ ATP in 30µl kinase buffer (50mM Tris-HCl pH 7.6, 10mM $MgCl_2$, 5mM DTT, 0.1mM spermidine, 0.1mM EDTA) for 30 min at 37°C. The radioactively labelled DNA was separated from unincorporated nucleotides by gel filtration through Biogel P60.

d. 5´ end labelling of oligonucleotides

20-200ng oligonucleotides were labelled with 10U polynucleotide kinase and

1.85MBq γ^{32}P ATP under the same conditions as for restriction fragments. A Nacs Prepac column was used to separate the oligonucleotides from unincorporated nucleotides following the manufacturer's instructions. The labelled oligonucleotides were precipitated with ethanol together with 10µg tRNA.

13. Construction of plasmids

a. cos 10.7 subclones

The 10kb Eco RI fragment containing most of the murine invariant chain (IN) coding sequences was cloned into the Eco RI site of pUC9 (Vieira and Messing, 1982) by H. J. Rahmsdorf (Rahmsdorf et al., 1986). The internal 3.9kb Hind III fragment from the 10kb Eco RI fragment was cloned into the Hind III site of pUC9 (Rahmsdorf et al., 1986) and was used as a probe in Northern blots. The 13kb Pst I-Hind III fragment which overlaps with the 10kb Eco RI fragment (see Fig. 1) was cloned into pSP64 vector of Melton et al. (1984). The 3kb Pvu II fragment which contains the IN RNA start site and the IN 5′ flanking sequences was cloned into the Hinc II site of pSP65 (cos10.7/6). See Figs. 1 and 12.

b. CAT plasmids

10.7/6 containing the start of transcription of the murine IN gene and about 1200bp 5′ flanking sequences was used as the starting material for constructs containing the 5′ flanking region of the murine IN gene linked to the bacterial chloramphenicol acetyl transferase gene (CAT). See Fig. 12.

Cos 10.7/6 was linearized with the restriction enzyme Nco I at position +85 of the IN gene and the 5′ overhangs (containing the ATG translation initiator codon) were removed by S1 nuclease digestion. A "fill-in" reaction was carried out with DNA polymerase I (Klenow fragment) to ensure the DNA was blunt-ended and Bam HI linkers were added to aid cloning. By digestion with Bam HI, the linkers were trimmed and the Bam HI site in the multiple cloning site (MCS) of the vector was cut (see Fig. 12). The resulting 1.3kb fragment containing the 5′ flanking sequences of the IN gene was cloned into the Bam HI site of pBLCAT3 (Luckow and Schütz, 1987). The orientation of the insert in pBLCAT3 was determined by a Bgl II

digestion. The resulting plasmid, called pIN-1200/+85CAT, contained 1200bp 5′ flanking sequences and 85bp of transcribed sequences from the murine IN gene.

A deletion mutant, (pIN-900/+85CAT), was constructed by digesting pIN-1200/+85CAT with Bgl II (which cuts at position -900 of the IN gene and in MCS (1) of pBLCAT3) and cloning the insert into the Bam HI site of pBLCAT3.

Further 5′ deletion mutants were constructed by Bal 31 digestion from the Hind III site of pIN-900/+85CAT, addition of Hind III linkers and after digestion by Hind III and Kpn I (Kpn I cuts behind the CAT gene in the second MCS of pBLCAT3, see Fig. 12), the insert containing the 5′ end of the murine IN gene was cloned into the Hind III and Kpn I sites of pBLCAT3.

This resulted in pIN-347/+85CAT; pIN-292/+85CAT; pIN-261/+85CAT; pIN-259/+85CAT; pIN-254/+85CAT; pIN-246/+85CAT and pIN-234/+85CAT. The construct pIN-189/+85CAT was made by digesting pIN-261/+85CAT with Stu I, which cuts at position -189 of the IN gene, adding Pst I linkers, digesting with Pst I and Kpn I, and ligating the relevant fragment into the Pst I and Kpn I sites of pBLCAT3.

3′ deletion mutants were made from pIN-261/+85CAT. This was linearized at the Stu I site at position -189 of the 5′ flanking region of the murine IN gene, Bal 31 digestion was carried out in the direction of the 5′ end of the IN sequences and Pst I linkers were added. After digestion by Pst I and Kpn I, the insert was ligated to pIN-189/+85CAT also digested with Pst I and Kpn I.

The resulting constructs were:

pIN-261/-203,-189/+85CAT; pIN-261/-210,-189/+85CAT; pIN-261/-215,-189/+85CAT and pIN-261/-235,-189/+85CAT.

pIN -249/-221,-189/+85CAT was made by ligating the synthetic oligonucleotide containing the sequences from -249 to -221 (see IV.10) of the murine IN gene into the plasmid pIN-189/+85CAT.

The double mutant (with the IRE in the inverted orientation) was made as follows: pIN-261/-215,-189/+85CAT was cut with Hind III, Pst I linkers were added, and then digestion with Pst I which also cuts at position -189 of the IN 5′ flanking region was carried out. The 46bp Pst I insert (-261/-215) was cloned into the Pst I site (at -189) of pIN-249/-221,-189/+85CAT. This resulted in pIN-249/-221, -215/-261,-189/+85CAT.

The orientation of the insert was determined by sequencing the construct (see below). The construct pIN-261/-215,■-189/+85CAT was made by cloning a 326bp Bam HI-Bgl II fragment from the 3′ untranslated region of the human collagenase gene (Angel et al., 1987), onto which Pst I linkers had been added, into the Pst I site of pIN-261/-215,-189/+85CAT.

Thymidine kinase (tk)-CAT constructs.

pIN-292/-73tkCAT construct was made by digestion of the plasmid pIN-292/+85CAT with Mae III which has a recognition site at position -73, addition of Bam HI linkers, digestion by Bam HI and Hind III and ligation of the fragment containing the 5′ flanking sequences (-292/-73) of the murine IN gene into the Bam HI and Hind III sites of pBLCAT2 (Luckow and Schütz, 1987). pIN-261/-189tkCAT was made by cutting the construct pIN-261/+85CAT with Stu I, which cuts at position -189, addition of Bam HI linkers, digestion by Bam HI and Hind III and ligation of the fragment containing the 5′ flanking sequences of the murine IN gene into the Bam HI and Hind III sites of pBLCAT2.

pIRE-SP65 was made by cloning the 46bp Hind III-Pst I fragment (positions -261 to -215) from pIN-261/-215,-189/+85CAT into the Hind III and Pst I sites of pSP65.

All constructs were controlled by appropriate restriction digests and sequencing the insert by the chain termination method (Sanger et al., 1977) as modified by Chen and Seeburg, (1985) using a primer which hybridizes to sequences directly 5′ to the Hind III site of pBLCAT3. The constructs, pIN-347/+85CAT; pIN-292/+85CAT and pIN-234/+85CAT were sequenced also by the Maxam and Gilbert method (1980) from the Hind III site of the vector which borders the 5′ end of the insert.

III. CELL CULTURE TECHNIQUES

1. Cell culture

Cells were maintained in a 37°C incubator with 6% CO_2.

GM637 were cultured in 9cm petri dishes with 10ml culture medium. Medium was changed every 3-4 days. Just before the cells became confluent, they were trypsinized

and reseeded at a lower density.

CH1.1 cells were maintained in suspension and kept at a density between 5×10^5 and 4×10^6/ml. They were diluted 1:5 with fresh medium every 3-4 days.

2. Trypsin treatment of cells

Culture medium was removed. Cells were washed with 3ml 0.25% trypsin and were then incubated with 1ml trypsin for 3-5 min at 37°C. They were resuspended in 9ml medium and replated at the required density, usually 1×10^6 cells per 9cm dish.

3. Maintenance of cell lines

Cell stocks were maintained in liquid N_2.

Logarithmically growing cells were trypsinized, centrifuged at 250×g and resuspended in 1ml culture medium containing 10% DMSO. After incubation on ice for 30 min, they were transferred to -80°C for several hours and finally to liquid N_2. Cells were thawed at 37°C, centrifuged 250×g in 10ml medium to remove the DMSO and plated out on petri dishes with fresh culture medium.

4. Stable Transfection

A modified form of the calcium phosphate/DNA coprecipitation method (abbreviation, calcium phosphate) was used (Graham and van der Eb, 1973; Spandidos and Wilkie, 1984).

Cells were plated out at a density of 1×10^6/9cm petri dish one day prior to transfection. 10µg of the relevant plasmid DNA and 1µg of pSV2neo (Southern and Berg, 1982) were transfected per dish. pSV2neo contains the gene coding for the enzyme Aminoglycoside-3´-phosphotransferase linked to the SV40 early region promoter, thereby allowing selection of stably transformed cells by their resistance to the toxic antibiotic G418. Cells transfected by the calcium phosphate method pick up a representative sampling of the various plasmids in the precipitate (Ausubel et al., 1987). DNA for transfection was coprecipitated with 125mM $CaCl_2$ in HBS (0.14M NaCl, 5mM KCl, 0.7M $Na_2HPO_4.2H_2O$, 0.5mM dextrose, 0.02M HEPES pH 7.05) in a volume of 0.5ml. The precipitate was left to stand at RT for 20 min, then added

dropwise to the cell medium. After 12-14 hours the precipitate was removed, the cells were washed twice with PBS (0.14M NaCl, 2.7mM KCl, 0.7mM $CaCl_2$ 6.5mM NaH_2PO_4, 1.5mM KH_2PO_4) and given fresh medium. 24 hours later, the cells were trypsinized and diluted 1:5 and plated out in selection medium (600µg/ml G418). G418 resistant colonies formed after 10-14 days. The cells were trypsinized and either collected for mass cultures or colonies were isolated and propagated as individual clones.

5. Transient transfection

a. calcium phosphate method

Cells were seeded at a density of 1×10^6 per 9cm petri dish one day before transfection. 10µg of plasmid DNA were transfected per dish and were coprecipitated with $CaCl_2$ in HBS buffer in the same way as for stable transfection. The precipitate was left on the cells for 8-12 hours after which the cells were washed twice with PBS and given fresh medium with or without IFN-γ.

b. DEAE-dextran method (Ausubel et al., 1987)

10^6 cells per 9cm petri dish were seeded one day before transfection. 10µg plasmid DNA were mixed with 0.5mg DEAE-dextran in 1ml TBS (25mM Tris-HCl pH 7.4, 137mM NaCl, 5mM KCl, 0.7mM $CaCl_2$, 0.5mM $MgCl_2$, 0.6mM Na_2HPO_4). Medium was removed from the cells which were then washed twice with TBS. 1ml of DNA mixture was added to the cells and left at RT for 30 min, shaking at intervals. Afterwards, cells were washed twice with TBS and given fresh medium. IFN-γ was added at this stage where necessary.

For both methods, cells were harvested 40-60 hours after transfection.

6. Preparation of RNA

a. Isolation of total RNA from cultured cells (Auffray and Rougeon, 1980)

Medium was removed from cells on 3-5 9cm petri dishes, they were washed twice with ice cold PBS and cells were harvested, with a rubber spatula, in 5ml PBS and were centrifuged at 670×g. The cell pellet was resuspended in 1ml PBS and an equal

volume of 2× PK buffer (0.2M Tris-HCl pH 7.5, 25mM EDTA, 0.3M NaCl and 2% SDS) was added. The released high molecular weight DNA was sheared by forcing the solution through a hypodermic syringe. 100µl 10mg/ml proteinase K was then added and the mixture was incubated at 37°C for 30 min. A phenol/chloroform extraction was carried out and an equal volume of 4M LiCl was added to the upper phase containing the nucleic acids. RNA was precipitated by overnight incubation at 4°C and was pelleted by centrifugation at 16800×g for 30 min. The RNA pellet was resuspended in 200µl H_2O and was reprecipitated with ethanol. After centrifugation at 10000×g, the RNA pellet was washed once with 80% ethanol, dried under vacuum and resuspended in H_2O. The concentration of the RNA was determined and the RNA was stored at -80°C.

b. Isolation of poly A^+ RNA from cultured cells.

Medium was removed from cells on 3-5 9cm petri dishes and the cells were washed twice with ice cold PBS. They were harvested, with a rubber spatula, in 10ml STE (20mM Tris-HCl pH 7.4, 10mM EDTA and 100mM NaCl) and SDS was added to a final concentration of 0.5%. The high molecular DNA was sheared by homogenising for 30 seconds with an ultra-turrax homogenizer. Proteinase K was added to a final concentration of 300µg/ml and the mixture was incubated at 37°C for 30 min. After bringing the final concentration of NaCl to 0.5M, 50mg oligo-dT cellulose (type VII) in HSB (20mM Tris-HCl pH 7.4, 10mM EDTA, 0.2% SDS and 300mM NaCl) was added. The poly A^+ RNA was allowed to bind to the oligo-dT cellulose overnight, rotating the mixture on a roto-tork.

The oligo-dT cellulose was then washed three times with HSB and the poly A^+ RNA was eluted from the cellulose three times, each with 1ml H_2O. 300µl of the eluent was used to determine the concentration of the RNA, the remainder was precipitated with ethanol. After overnight incubation at -20°C, the RNA was pelleted by centrifugation at 10000×g, the RNA pellet was washed once with 80% ethanol, dried under vacuum and resuspended in H_2O, and the RNA was stored at -80°C.

c. Isolation of mouse spleenocyte poly A^+ RNA

The spleen was removed after cervical dislocation. It was washed once in PBS and single cells were obtained by forcing the spleen through a fine grid. The cells were harvested in PBS and washed again. They were resuspended in 10ml STE and the isolation of the poly A^+ RNA continued as in b above.

7. Preparation of cell extracts for CAT-assay

The medium was removed from one 9cm petri dish and the cells were washed twice with PBS. The cells were harvested in 1ml PBS with a rubber spatula, were centrifuged for 5 min at 5600×g and resuspended in 100µl 250mM Tris-HCl pH 7.8. They were lysed by rapid freezing (ethanol/dry ice bath) and thawing (37°C, 5 min) - this was repeated three times. The cell debris was pelleted by centrifugation for 10 min at 5600×g and the supernatant containing protein was stored at -20°C.

The protein concentration was determined by the method of Lowry et al. (1951) as follows: 10µl cell extract were mixed with 990µl solution I (2% Na_2CO_3 in 0.1M NaOH), 2ml freshly prepared solution IV (100ml solution I, 1ml 2% NaKTartrate and 1ml 1% $CuSO_4$) was added and the mixture was incubated at RT for 10 min. Then 200µl Folin and Ciocalteu's phenol reagent (50% in H_2O) was added. After incubation for a further 30-40 min at RT, the extinction coefficient at 735nm was measured. Parallel, standard samples (BSA) of known concentration were measured to obtain a standard curve from which the concentration of protein in the test sample was calculated.

8. Preparation of nuclear extracts (Dignam et al., 1983)

1-3 $\times 10^8$ GM637 cells were grown to about 80% confluency in 15cm petri dishes. Cell medium was removed, the cells were washed twice with ice cold PBS and were harvested in cold PBS. All following steps were carried out at 4°C. The cells were centrifuged at 350×g, washed once with PBS and centrifuged again. The volume of the cell pellet was determined (packed cell volume = PCV). The cells were resuspended in 5× PCV buffer A (10mM HEPES-KOH pH 7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT) and incubated on ice for 10 min. The cells were centrifuged at

5000×g for 10 min and were resuspended in 2× PCV buffer A and broken open in a Dounce homogenizer to release the cell nuclei. The latter were pelleted by centrifugation at 2500×g for 10 min. The nuclei were then resuspended in buffer C (20mM HEPES-KOH pH 7.9, 25% glycerol, 1.5mM $MgCl_2$, 0.42M NaCl, 0.2mM EDTA, 0.5mM DTT and 1mM PMSF) at a concentration of 250µl buffer C/10^8 cells. The nuclei were broken open by 10 strokes of a Dounce homogenizer, the suspension was transferred to a glass beaker and then stirred for 30 min on a magnetic stirrer. The proteins were precipitated by the addition of $(NH_4)_2SO_4$ (0.33g/ml buffer C). The pH was adjusted to 7.0 with NaOH. After 2 hours, the proteins were pelleted by centrifugation at 16800×g for 30 min and were resuspended in 400µl buffer D (20mM HEPES-KOH pH 7.9, 20% glycerol, 2mM $MgCl_2$, 40mM KCl, 0.2mM EDTA, 0.5mM DTT and 1mM PMSF) and dialysed for 5 hours against buffer D with three buffer changes. The dialysed extract was centrifuged in an eppendorf centrifuge for 5 min, aliquoted in small amounts and stored in liquid N_2.

IV. ANALYTICAL METHODS

1. CAT-assay (Gorman et al., 1982)

2µl ^{14}C chloramphenicol were added to the required amount of cell extract (III.7) in 250mM Tris-HCl pH 7.8 in a final volume of 80µl. The reaction was started by the addition of 20µl 4mM acetyl coenzyme A and proceeded at 37°C for 2 hours. The reaction was stopped by the addition of 1ml ethyl acetate. The chloramphenicol was extracted from the aqueous phase with ethyl acetate by vortexing and centrifugation for 5 min at 5600×g. The ethyl acetate was evaporated in a vacuum centrifuge and the chloramphenicol was resuspended in 15µl ethyl acetate and dotted onto a TLC sheet. Chloroform/methanol (9:1) was used as the solvent in the chromatography. The acetylated chloramphenicol was separated from the unacetylated chloramphenicol within 1 hour, after which the TLC sheet was air dried and exposed to Kodak XAR-5 film at -80°C.

To calculate the amount of chloramphenicol acetylated, the unacetylated and

acetylated forms were cut out and placed in counting vials with 5ml quickzint and the cpm were determined in a scintillation counter. The specific CAT activity in pmol $mg^{-1}min^{-1}$ was determined from the equation:

$$\text{Specific activity} = \frac{7551\text{pmol chloramphenicol} \times \text{cpm acetylated chloramphenicol}}{120\ \text{min} \times \text{mg protein extract} \times \text{cpm input chloramphenicol.}}$$

2. S1 nuclease analysis (Berk and Sharp, 1977)

20µg total RNA or 2-3µg poly A^+ RNA were precipitated with 20-50fmol 5′ end-labelled S1 probe (see II.12c) with ethanol. After washing the precipitate with 80% ethanol and drying under vacuum, the nucleic acids were dissolved in 10µl hybridization buffer (80% deionised formamide, 40mM PIPES pH 6.4, 1mM EDTA and 0.4M NaCl) and were denatured for 5 min at 85°C and hybridized overnight at 47°C. S1 digestion was carried out by the addition of 50U S1 nuclease in 100µl cold S1 buffer (300mM NaCl, 30mM CH_3COONa pH 4.5, 3mM $ZnSO_4$ and 10µg/ml denatured salmon sperm DNA). The reaction was carried out at 30°C for 90 min and was stopped by a phenol/chloroform extraction and the reaction products were precipitated with ethanol. After centrifugation at 10000×g, the pellet was washed once with 80% ethanol and dried under vacuum. The precipitate was resuspended in 3µl SSB and the reaction products were analysed on a denaturing polyacrylamide gel (see I.3c).

3. DNA sequencing

a. Chemical cleavage method of Maxam and Gilbert (1980)

The sequencing kit from New England Nuclear, Dreieich, was used.

20pmol end-labelled DNA were dissolved in 40µl H_2O and were divided for the different reactions into 5 eppendorf tubes each containing 8µg carrier DNA (denatured salmon sperm).

G+A reaction

13µl H_2O and 2µl piperidine formate were added to 10µl DNA and were incubated at 37°C for 30 min. The reaction mix was cooled to -70°C, condensed in a vacuum

centrifuge, resuspended in 20µl H_2O and after cooling to -70°C, lyophilized again.

G reaction

200µl G reaction buffer and 1µl DMS were added to 5µl DNA and were incubated at RT for 2 min. The reaction was stopped by the addition of 50µl G stop solution and the DNA was precipitated with ethanol.

A+C reaction

10µl DNA were mixed with 100µl 1.2M NaOH, 1mM EDTA, heated for 10 min at 90°C and then neutralized with 150µl 1M acetic acid. 4µg tRNA were added and the DNA was precipitated with ethanol.

C reaction

15µl 5M NaCl and 30µl hydrazine were added to 5µl DNA and incubated for 10 min at RT. The reaction was stopped by the addition of 200µl hydrazine-stop solution and the DNA was precipitated with ethanol.

C+T reaction

10µl H_2O and 30µl hydrazine were added to 10µl DNA and incubated for 10 min at RT. The reaction was stopped by the addition of 200µl hydrazine-stop solution and the DNA was precipitated with ethanol.

The reaction products from b, c, d and e were centrifuged at 8700×g, the precipitate resuspended in 0.3M sodium acetate pH 4.8 and precipitated again with ethanol. After centrifugation, the precipitate was washed twice with 80% ethanol and the pellet was dried.

The DNA from all reactions was then resuspended in 100µl 1M piperidine, incubated for 30 min at 90°C and evaporated overnight in a vacuum centrifuge. The precipitate was resuspended twice in 20µl H_2O, evaporating the H_2O each time in a vacuum centrifuge. The DNA was resuspended in 10µl SSB and the reaction products analysed on a denaturing polyacrylamide gel (see I.3c).

b. Sequencing double stranded plasmid DNA by the chain termination method of Sanger et al. (1977) as modified by Chen and Seeburg, (1985).

20µg purified plasmid DNA were incubated with 100µg/ml RNase in 10mM Tris-HCl pH 7.5, 1mM EDTA for 15 min at RT. The DNA was then extracted with

phenol/chloroform and precipitated with ethanol. After centrifugation, the precipitate was washed twice with 80% ethanol and the pellet was dried. The DNA was resuspended in 20µl H_2O and the concentration determined. 2µg DNA were denatured in 40µl denaturing buffer (0.2M NaOH, 0.2mM EDTA) for 5 min at RT. 4µl 2M CH_3COONH_4 pH 4.5 was added to neutralize the solution and the DNA was precipitated with ethanol. After centrifugation, the precipitate was washed once with 80% ethanol and the DNA was dried.

The denatured DNA was then incubated with 5µl primer (0.5pmol/µl), 1.5µl 10× annealing buffer (70mM Tris-HCl pH 7.5, 70mM $MgCl_2$, 300mM NaCl, 1mM EDTA and 100mM DTT), 2µl (0.6MBq) $\alpha^{35}S$ dATP and 6.5µl H_2O. While this hybridization was taking place at 37°C for 15 min, the respective dNTP/ddNTP solutions were prepared in four eppendorf tubes. 2U DNA polymerase I (Klenow fragment) were added to the hybridization mixture and 3µl of the resulting mix were added to each of the prepared eppendorf tubes. Incubation was carried out at 30°C for 30 min. Following the addition of 1.5µl chase solution (0.125mM dNTPs), the samples were incubated at 30°C for a further 15 min. Finally, the samples were lyophilized in a vacuum centrifuge and resuspended in 4µl SSB and the reaction products analysed on a denaturing polyacrylamide gel (see I.3c).

The concentrations of the dNTP/ddNTP solutions in annealing buffer, are as follows:

A-mix	100 µM	dTTP	C-mix	100 µM	dTTP
	100 µM	dCTP		10 µM	dCTP
	100 µM	dGTP		100 µM	dGTP
	100 µM	ddATP		100 µM	ddCTP
G-mix	100 µM	dTTP	T-mix	5 µM	dTTP
	100 µM	dCTP		100 µM	dCTP
	5 µM	dGTP		100 µM	dGTP
	120 µM	ddGTP		500 µM	ddTTP

4. Primer extension analysis of RNA (McKnight and Kingsbury, 1982)

A synthetic oligonucleotide primer (see below) with sequence complementary to the RNA being analysed was end-labelled (II.12). 2ng radioactively labelled primer were dried down with 2-3µg poly A tailed RNA and were resuspended in 20µl hybridization buffer (250mM KCl, 10mM Tris-HCl pH 7.5, 1mM EDTA). Hybridization was carried out for 1 hour at 60°C. The samples were then chilled on ice for 2 min and then at RT 40µl reverse transcriptase mix were added. This mix contained for 10 samples: 120µl nucleotide mix (2.5mM dNTPs), 120µl actinomycin D (250µg/ml), 80µl 5× reverse transcriptase buffer (375mM Tris-HCl pH 7.5, 75mM DTT, 60mM $MgCl_2$), 4µl RNase inhibitor (120U) and 10µl M-MLV reverse transcriptase (5U/µl). The reaction took place at 37°C for 30-45 min after which the nucleic acids were precipitated with ethanol. After centrifugation at 10000×g, the pellet was washed once with 80% ethanol and dried under vacuum. The precipitate was resuspended in 3µl SSB and the reaction products analysed on a denaturing polyacrylamide gel (see I.3c).

Sequencing RNA by primer extension

The reaction was carried out as for primer extension but with the addition of the corresponding dideoxynucleotides to a final concentration of 1.2mM (or 0.7mM also for ddATP).

Primer used in primer extension with sequencing of the transfected murine IN gene in fibroblasts:

$_{+262}$**GCCCTGTTGCTGGTACAG**$_{+244}$

Primer used in the analysis of IN RNA from different murine strains:

$_{+126}$**GGCAACTGTTCATGGT**$_{+110}$

5. Nuclear run-on assay (modified from Kazmaier et al., 1985)

Isolation of nuclei:

1-2×10^7 cells were harvested in cold PBS and centrifuged at 250×g at 4°C for 5 min. After washing once with cold PBS, the cells were resuspended in 2ml lysis buffer

(10mM Tris-HCl pH 7.9, 140mM KCl, 5mM $MgCl_2$, 1mM DTT) and Nonidet P-40 was added to a final concentration of 0.5%. Incubation took place on ice for 10 min shaking occasionally to break open the cells. The nuclei were pelleted by centrifugation for 3 min at 350×g at 4°C.

Transcription reaction:

The nuclei were resuspended in 2ml reaction buffer (20mM Tris-HCl pH 7.9, 140mM KCl, 10mM $MgCl_2$, 1mM DTT) and were incubated on ice for 5 min. Following centrifugation at 350×g for 3 min, the nuclei were resuspended in 100µl reaction buffer containing 4mM each of GTP, ATP and CTP, 10mM phosphocreatine, 2U creatine phosphokinase and 3.7MBq α^{32}P UTP. Incubation took place at 26°C for 20 min. The reaction was stopped by the addition of 1ml high salt buffer (10mM Tris-HCl pH 7.4, 0.5M NaCl, 50mM $MgCl_2$, 2mM $CaCl_2$ and 50µg tRNA), which lyses the nuclei. RNase free DNase was added to a final concentration of 50µg/ml, and the suspension was incubated at 37°C until it was no longer viscous (2-10 min). After adjusting the incubation mix to 10mM EDTA and 1% SDS, proteins were degraded by proteinase K (final concentration of 1.4mg/ml) for 30 min at the same temperature. RNA was extracted three times with phenol/chloroform, was ethanol precipitated and redissolved in 1mM UTP, 30mM $Na_4P_2O_7$. Unincorporated nucleotides were removed by precipitation of the RNA with 10% trichloroacetic acid (TCA) for 20 min on ice. RNA was pelleted by centrifugation at 5600×g for 15 min. The pellet was washed three times with 500µl 5% TCA, 10mM $Na_4P_2O_7$. TCA was neutralized by redissolving the pellet in 200µl 0.1M CH_3COONa pH 7.0, and the RNA was recovered by ethanol precipitation.

Fixing DNA to nitrocellulose:

2-10µg DNA were resuspended in 200µl TE (10mM Tris-HCl pH 7.5, 1mM EDTA). 50µl 1M CH_3COONa pH 4.0 was added and the DNA was incubated at 55°C for 15 min, then transferred immediately to ice. After the addition of 125µl 1M NaOH, incubation took place at RT for 20 min. Finally, 110µl 1M HCl, 300µl 20× SSC (1× SSC is 0.15M NaCl, 0.75M sodium citrate), 240µl TE and 1µl 10mg/ml ethidium

bromide were added. The denatured DNA was then dotted onto a nitrocellulose filter which had been preincubated in H_2O for 1 min and 6× SSC for 10 min. The filter was then incubated in 6× SSC, 0.05% SDS for 5 min, air dried and baked at 80°C for 2 hours.

Hybridization

The filter was prehybridized with 3-10ml hybridization solution (0.6M NaCl, 0.2M Tris-HCl pH 8.0, 20mM EDTA, 50% formamide, 0.2% each BSA, ficoll and polyvinyl pyrollidone, 2.7% $Na_4P_2O_7$, 0.2% SDS, 0.25mg/ml tRNA and 0.115mg/ml calf thymus DNA) for 2 hours at 42°C. The radioactively labelled RNA was resuspended in 200µl hybridization solution, depurinated calf thymus DNA was added to a final concentration of 0.5mg/ml and the hybridization mixture was added to the filter sealed in a plastic bag. Hybridization took place at 42°C for 48 hours. The filter was washed twice for 30 min at 70°C with 2× SSC, 0.2% SDS, 10µg/ml calf thymus DNA and then three times at 42°C with 2× SSC, 0.2% SDS, each for 45 min. The filter was air dried and exposed to Kodak XAR-5 film with an intensifying screen at -80°C.

6. RNA dot blot (Kafatos et al., 1979)

Between 1 and 4µg RNA were dried in a vacuum centrifuge, resuspended in 20µl FF (50% formamide, 6% formaldehyde in TBE (see I.3)) heated at 37°C for 15 min and then at 65°C for 3 min, were diluted with 180µl 10× SSC and then spotted onto nitrocellulose filters using a hybrid-dot blot apparatus (BRL). The filter was then washed in 6× SSC, 0.05% SDS for 5 min, and dried as described in IV.5.

Hybridization of a nick-translated DNA probe to the filter was carried out as follows: The filter was prehybridized for 2 hours at 65°C in 4× SSC, 0.02% of each BSA, ficoll and polyvinyl pyrollidone, 0.05% $Na_4P_2O_7$, 6.7mM NaH_2PO_4, 10mM Na_2HPO_4, and 0.1% SDS. To block unspecific hybridization, small denatured salmon sperm DNA was added in the second hour of prehybridization to a final concentration of 20µg/ml. The hybridization mix contained 1-3×10^6 cpm/ml of nick-translated DNA (denatured at 90°C for 10 min) in 4× SSC, 10mM EDTA, 0.1% SDS and salmon sperm DNA (20µg/ml). The volume of hybridization solution was determined from

the area of the filter in $cm^2 \times$ 13µl (volume required to saturate $1cm^2$) plus 30%. Hybridization was carried out for 16 hours at 65°C. The filter was washed 4 times at 65°C in 2× SSC, 1× SSC, 1× SSC and 0.5× SSC respectively, each containing 0.05% $Na_4P_2O_7$, 6.7mM NaH_2PO_4, 10mM Na_2HPO_4, and 0.1% SDS. The filter was sealed in a plastic bag and exposed wet to Kodak XAR-5 film at -80°C with intensifying screen. Dot blot quantitation was carried out by scanning autoradiograms in the linear exposure range of the film in a vertical and horizontal direction with a Joyce-Loebel densitometer.

7. Northern blot hybridization (McMaster and Carmichael, 1977)

The required amount of RNA was dried in a vacuum centrifuge and resuspended in 5µl H_2O and 15µl denaturing buffer (50µl 20× PB (0.2M NaH_2PO_4, 10mM Na_2HPO_4, pH 6.85), 500µl DMSO and 195µl 30% deionised glyoxal). The samples were heated to 50°C for 5 min, cooled rapidly on ice and 5µl RNA loading buffer (50% glycerol, 1× PB, 0.1% bromophenol blue) were added.

The RNAs were then loaded onto a 1% vertical agarose gel made in 1× PB. Electrophoresis took place at 100V at 4°C for 3 hours. The gel was stained with acridine orange (30µg/ml) in 1× PB and the RNA was visualised under short wave (260nm) UV-light. The RNAs were transferred to a nitrocellulose filter using the apparatus described in Maniatis et al. (1982), by the method of Southern, (1975). The filter was dried, baked, hybridized and analysed as described in IV.6.

8. *In vitro* transcription with SP6 polymerase (Melton et al., 1984)

5µg pSP64 plasmid DNA were linearized with a restriction enzyme that does not cut between the SP6 promoter and the insert. Phenol/chloroform extraction and ethanol precipitation were carried out to purify the DNA which was finally resuspended in 16.5µl H_2O. The following substances were added at RT: 10µl transcription buffer (200mM Tris-HCl pH 7.5, 30mM $MgCl_2$, 10mM spermidine), 0.5µl 1M DTT, 2µl RNasin (25U/µl), 10µl 2.5mM each of ATP, TTP and GTP, 3.7MBq α^{32}P UTP (10µl) and 1µl SP6 polymerase (9U/ml). Transcription took place at 40°C for 60 min. The DNA template was removed by incubation with DNase I (12.5µg/µl) at 37°C for 15

min. The synthesized RNA was purified by phenol/chloroform extraction, precipitated with ethanol and hybridized as in IV.5, to RNAs separated by gel electrophoresis under denaturing conditions and fixed to nitrocellulose as in IV.7.

9. Electrophoresis mobility shift assay (Barberis et al., 1987)

An α^{32}P end-labelled oligonucleotide (see II.2) was dissolved in DNA binding buffer (10mM HEPES pH 7.9, 60mM KCl, 4% ficoll, 1mM DTT, 1mM PMSF). 4µg nuclear extract (III.8) were incubated with 1fmol oligonucleotide in a final volume of 20µl. As unspecific competitor, poly (dIdC) was added at a final concentration of 0.1µg/µl. The individual components were added in the following order: DNA binding buffer; poly (dIdC); competitor oligonucleotide (when present); nuclear extract; labelled oligonucleotide. Incubation took place at RT for 20 min, after which the reaction products were separated on a 4% native polyacrylamide gel (as section I.3b except that the gel and electrophoresis buffer was 0.25× TBE). Electrophoresis took place for 90 min at 100 volts. The gel was fixed for 10 min in 10% acetic acid, dried onto Whatman filter paper and exposed to Kodak XAR-5 film at RT.

10. Oligonucleotides used in electrophoresis mobility shift assay.

Bases in normal print correspond to sequences of restriction enzyme recognition sites, bases in bold print correspond to sequences from the murine IRE.

IRE: -249/-221

AGCTT**TCCAGAAGTCTGCCTAGAAACAAGTGATG**G

A**AGGTCTTCAGTCGGATCTTTGTTCACTAC**CCTAG

IRE A: -247/-238

AGCTT**CAGAAGTCTG**CTGCA

A**GTCTTCAGAC**G

IRE B: -237/-223

AGCTT**CCTAGAAACAAGTGA**CTGCA

A**GGATCTTTGTTCACT**G

mIRE (mutated IRE -248/-221)

AGCTT**CCATCGATCTGAGTCTAAACAAGGATTG**CTGCA

A**GGTAGCTAGACTCAGATTTGTTCCTAAC**G

RESULTS

I. INTERFERON-γ INCREASES THE TRANSCRIPTION OF THE TRANSFECTED MURINE INVARIANT CHAIN GENE

In order to investigate the regulation of the expression of the murine IN gene by IFN-γ and to identify the cis-acting elements involved, the gene was cloned and analysed by transfection analysis for its response to IFN-γ. Essential for this analysis were cells which have a high transfection efficiency and a distinguishable endogenous IN gene. The human skin fibroblast cell line, GM637, was chosen because it fulfilled these properties. The human and murine IN genes were known to be about 70% homologous but probes exist which do not cross-hybridize under stringent conditions. Also necessary for this analysis is a pure form of IFN-γ. Human IFN-γ cloned into and prepared from *E. coli* was used (Gray et al., 1982; Gray and Goeddel, 1987).

a. Interferon-γ induces an increase in mRNA expression from the transfected invariant chain gene.

The murine IN gene was identified and cloned by M. Steinmetz, Basel, by screening a cosmid genomic library prepared from murine DNA from the AKR strain, with a murine IN cDNA clone isolated by P. Singer, Heidelberg (Singer et al., 1984). The cosmid containing the entire murine IN gene, cos 10.7, was mapped with 11 restriction enzymes and the location of the IN gene was determined by Southern blot hybridization against the IN cDNA probe (Yamamoto et al., 1985b). Fig. 1 shows a map of cos 10.7 and two subclones. Relevant restriction sites of the subclones are indicated.

Cos 10.7, together with pSV2neo was transfected into GM637 cells by the calcium phosphate method (see methods III.4). Stably transfected clones were selected by G418 resistance. Mass cultures and single clones were analysed for IFN-γ regulated expression of the murine IN gene by analysis of mRNA levels in the

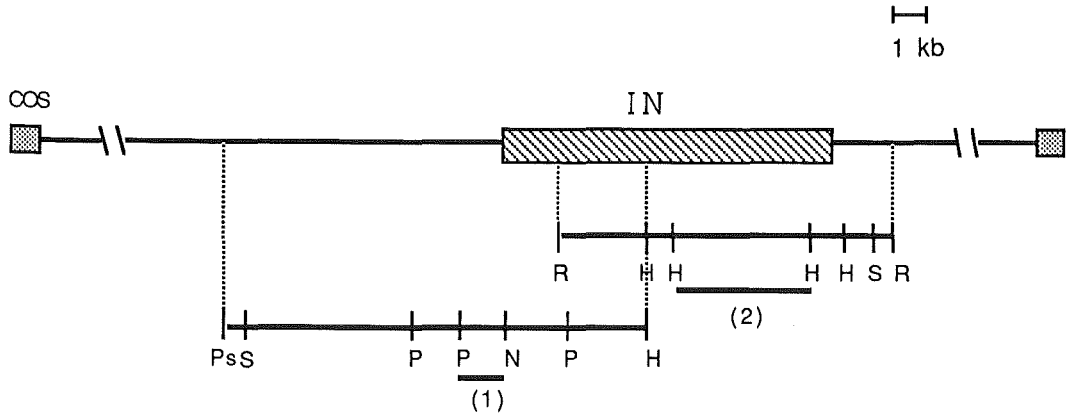


Fig 1. Map of cos 10.7 which contains the murine IN gene.
The location of the IN gene was determined by Southern blot analysis, (Yamamoto et al., 1985b). Two subclones, a 13kb Pst I-Hind III fragment and a 10kb Eco RI fragment are shown and their relevant restriction enzyme sites are indicated. Restriction maps were constructed by partial digestion of end-labelled DNA and by multiple digestions of the DNA. The restriction sites are: R = Eco RI, H = Hind III, N = Nco I, P = Pvu II, Ps = Pst I, S = Sph I. The two fragments indicated, 1 and 2 were used in different hybridization experiments: 1 = S1 probe (1.3kb Pvu II-Nco I), 2 = probe used in northern blots (3.9kb Hind III fragment).

transfected cells. Total RNA was prepared from transfected cells either untreated (control) or treated with IFN-γ (100U/ml) for 48 hours and was dotted directly onto a nitrocellulose filter. The filter was hybridized with a probe from the murine IN gene, the 3.9kb Hind III fragment (Fig. 1), which was radioactively labelled by nick-translation. The amount of radioactive probe that hybridizes to the RNA is a measurement of the amount of IN mRNA present in a particular sample. The results, shown in Fig. 2, indicate that indeed the expression of the transfected murine IN gene is regulated by IFN-γ resulting in an elevated level of IN specific RNA in IFN-γ treated cells compared to untreated cells. In Fig. 2A, the response of a mass culture of at least 50 single clones to IFN-γ is shown. The expression of the endogenous human gene was also analysed in the same experiment by hybridization of the RNAs with a human cDNA probe (Rahmsdorf et al., 1986). As expected, IFN-γ treatment caused an induction of expression of the endogenous IN gene (Fig. 2A). Also worth noting is the fact that RNA from cells transfected only with pSV2neo hybridizes only to the

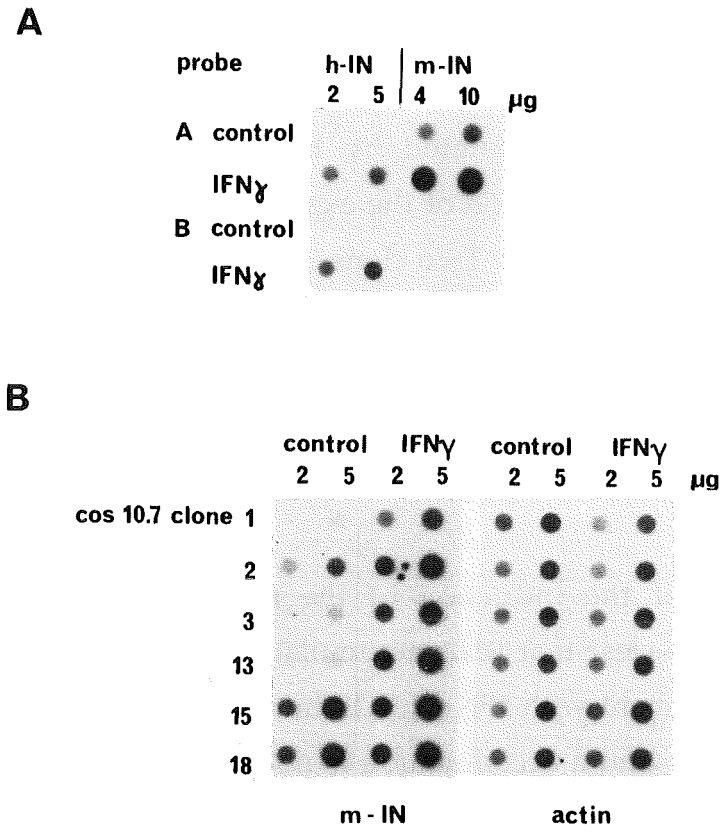


Fig 2. The expression of the transfected murine invariant chain gene is induced by IFN-γ in human fibroblasts.
2A: A mass culture of at least 50 independent GM637 clones transfected with cos 10.7 and pSV2neo (A), or a mass culture of the same cells transfected with pSV2neo alone (B) were treated with IFN-γ (100U/ml). After 72 hours, RNA was prepared and the indicated amounts were probed with a human cDNA probe (Rahmsdorf et al., 1986) or with a mouse IN specific probe (3.9kb Hind III fragment; Fig 1). Control: RNA from untreated transfected cells.
2B: Single GM637 clones transfected with cos 10.7 and pSV2neo were treated with IFN-γ (100U/ml). After 72 hours, RNA was prepared and the indicated amounts were probed with the murine IN specific probe (3.9kb Hind III fragment; Fig 1) or with an α-actin specific probe (Minty et al., 1981). Control is RNA from untreated cells.

human probe but not to the murine probe. This shows that the probe for the murine IN does not cross-hybridize to human IN RNA. The analysis of single clones is shown in Fig. 2B. There was some variability in expression and inducibility by IFN-γ between the different clones which is probably due to the different locations of the gene in the transfected cells. The same RNAs were hybridized against a probe for the actin gene (Minty et al., 1981), whose expression is not influenced by IFN-γ treatment (Fig. 2B). This was used as a

control that the same amount of RNA was dotted in each sample.

The following conclusions can be made from this experiment: 1) The expression of the murine IN gene is regulated by human IFN-γ. 2) The sequences responsible for the IFN-γ regulation of the expression of the murine IN gene are contained within the cosmid 10.7. Two clones whose expression of murine IN is well induced by IFN-γ were chosen for further study. These were cos 10.7 clones 1 and 2 (Fig. 2B).

b. Cycloheximide inhibits the induction of invariant chain mRNA levels by interferon-γ

When the kinetics of the induction of the murine IN gene by IFN-γ are examined (Fig. 3), it is clear that RNA accumulates over a long period of time: in cos 10.7 clone 1, treatment with IFN-γ for 10 hours results in a two-fold increase of IN mRNA, but maximum accumulation is not reached until 48-72 hours after IFN-γ treatment. The experiment does not indicate whether there is a short lag phase in the induction. To investigate this point, the mechanism by which IFN-γ activates the murine IN gene was analysed.

There are two possible mechanisms whereby IFN-γ treatment could lead to an increase in murine IN mRNA levels: it could either directly cause the accumulation of murine IN mRNA without the synthesis of intermediary proteins as is the case for several IFNs α and β induced genes (Friedman et al., 1984, Faltynek et al., 1985; Hannigan and Williams, 1986) or a mechanism in which the synthesis of one or more intermediate proteins could be involved. To distinguish between these possibilities the clone cos 10.7 clone 1 was analysed for IFN-γ inducibility in the presence or the absence of the protein synthesis inhibitor, cycloheximide. If protein synthesis is required for the activation, then the induction of IN expression by IFN-γ should be inhibited in the presence of cycloheximide.

RNA was prepared from cells treated for 24 hours either with IFN-γ alone (100U/ml), with cycloheximide alone (10μg/ml) or with a combination of both. At this concentration, cycloheximide inhibits about 95% protein synthesis (experiment not shown). The RNA was dotted directly onto a nitrocellulose

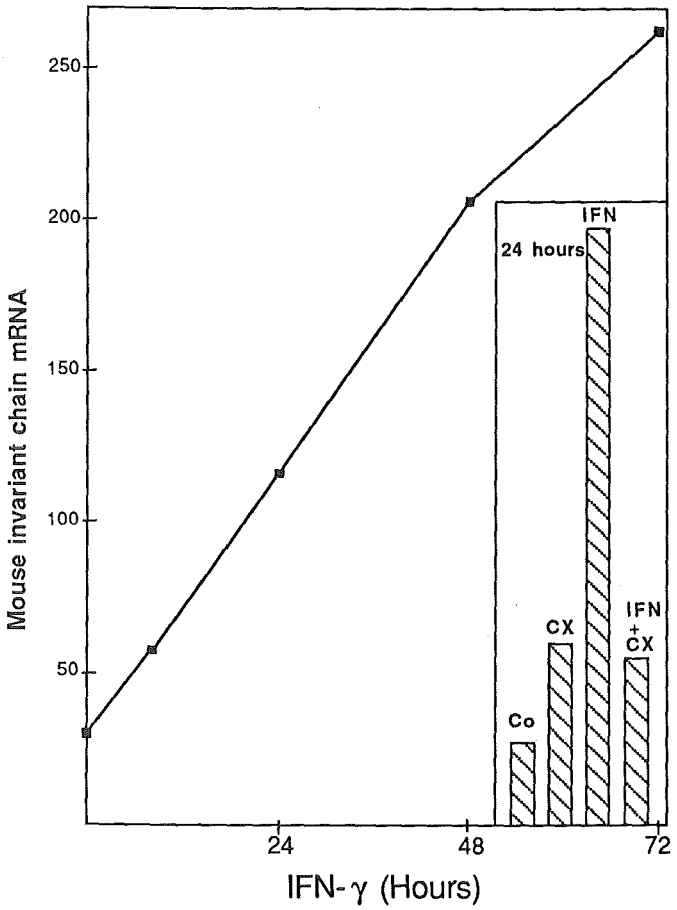


Fig. 3. Kinetics of IN mRNA accumulation in cells treated by IFN-γ and inhibition of this accumulation by cycloheximide.
RNA from cos 10.7 clone 1 cells treated for the indicated times with 100U/ml IFN-γ was dotted onto a nitrocellulose filter and probed with the mouse IN specific probe (3.9kb Hind III fragment; Fig 1). Dots were evaluated by densitometric scanning. Arbitrary units are used. Inset: The same cells were treated for 24 hours with IFN-γ (100U/ml), or with cycloheximide (10μg/ml) or with a combination of both. RNA was evaluated as above. Co: control; CX: cycloheximide; IFN: IFN-γ.

filter which was hybridized with a probe specific for the murine IN gene. As shown in Fig. 3 inset, cycloheximide alone causes a twofold increase in IN mRNA levels. Even so, the increase caused by IFN-γ alone (8 fold) drops in the presence of cycloheximide to the level reached by cycloheximide alone. Thus, protein synthesis is necessary for activation of murine IN gene expression by IFN-γ.

c. Interferon-γ enhances the transcription of the invariant chain gene but does not prolong the half-life of its mRNA.

IFN-γ could cause murine IN mRNA accumulation, either by inducing the transcription of the IN gene or by stabilizing the IN mRNA or by a combination of these mechanisms. These possibilities were investigated as follows:

To investigate whether IFN-γ increases the transcription of the murine IN gene a "nuclear run-on" experiment was carried out. Nuclei were isolated from cos 10.7 clone 2 cells either untreated or treated for different times with IFN-γ. An *in vitro* transcription followed in the presence of radioactively labelled UTP under conditions where preexisting transcripts were completed but new transcripts were not initiated. Cells actively transcribing a specific gene have many polymerase molecules attached to that gene so that more RNA transcripts are produced than in cells not transcribing or transcribing the gene at a lower rate. The radioactively labelled RNA produced was assayed quantitatively by hybridization to an excess of specific DNA probe fixed to a nitrocellulose membrane. The amount of radioactivity bound to the probe quantitates the level of transcription of the gene being probed. This experiment was carried out with cells treated for 0, 4 or 24 hours with IFN-γ and showed that the transcription of the murine IN gene is elevated in cells treated with IFN-γ for 4 hours as compared to untreated cells (Fig. 4). After 24 hours exposure to IFN-γ, the transcription rate drops slightly. The transcription of the actin gene was not influenced by IFN-γ treatment. I concluded from this experiment that IFN-γ induces the expression of the murine IN gene by activating its transcription.

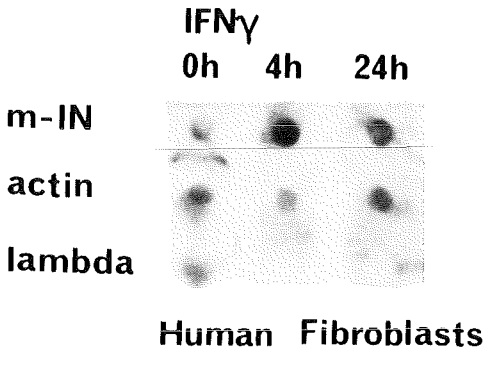


Fig. 4. Transcriptional regulation of the murine invariant chain RNA accumulation by IFN-γ.
Cos 10.7 clone 2 transfectants were treated for the indicated times with 100U/ml IFN-γ. Nuclei were isolated and incubated with $\alpha\,^{32}P$ UTP. Radioactively labelled RNA was isolated and hybridized to the indicated probes of which 4μg specific DNA was fixed to nitrocellulose. Murine and actin probes are as described (Fig 2). Lambda: DNA from the bacteriophage lambda, as a negative control. An equal amount of TCA precipitable activity was used in each hybridization.

It is possible that IFN-γ prolongs the half-life of the IN mRNA in addition to activating transcription of the gene. Such a situation is known for other inducers of gene expression, for example, steroid hormones which enhance both the transcription and the half-life of the mRNAs of responding genes (Shapiro and Brock, 1985, and references therein, Spindler et al., 1982). This possibility was investigated by measuring the half-life of the murine IN mRNA in the presence of a transcription inhibitor, following maximum induction by IFN-γ treatment. If IFN-γ stabilizes the IN mRNA, then the IN mRNA half-life is longer in cells treated with inhibitor plus IFN-γ than in cells treated with the inhibitor alone. Actinomycin D which inhibits transcription by binding to the DNA and preventing movement of the RNA polymerase, was used.

The influence of IFN-γ on the half-life of the murine IN mRNA was analysed as follows: cos 10.7 clone 1 was treated with IFN-γ (400U/ml) for 48 hours to achieve maximum IN RNA accumulation (Fig. 5, lane 2). After extensive washing to remove IFN-γ, the cells were treated further for 4, 12, or 24 hours with either actinomycin D (5μg/ml) or actinomycin D plus IFN-γ (400U/ml). Poly A^+ RNA was prepared, separated by agarose gel electrophoresis, transferred to nitrocellulose and hybridized against a probe for the murine IN gene. Actinomycin D alone does not induce expression of the murine IN gene but completely inhibits the IFN-γ induced transcription of the gene (Fig. 5, lane 3). After addition of actinomycin D to IFN-γ treated cells for 4 hours, IN mRNA is still present (Fig. 5, lanes 4 and 5) but by 12 hours treatment, decay of the RNA is obvious (Fig. 5, lanes 6 and 7). After 24 hours treatment with actinomycin D, almost all of the IN mRNA is totally degraded (Fig. 5, lanes 8 and 9). IFN-γ does not prolong the half-life of the murine IN gene since there is no difference between the length of the IN mRNA half-life in the presence or in the absence of IFN-γ. By densitometric scanning of the autoradiogram and plotting a graph (Fig. 5), the half-life of the IN mRNA was estimated to be about 6 hours.

In conclusion, IFN-γ induces the transcription of the murine IN gene but does not influence the half-life of the IN mRNA. As the next step towards the goal of

analysing the sequences responsible for the transcriptional regulation of the murine IN gene by IFN-γ, I determined the structure and the sequence of the 5′ flanking region of the gene.

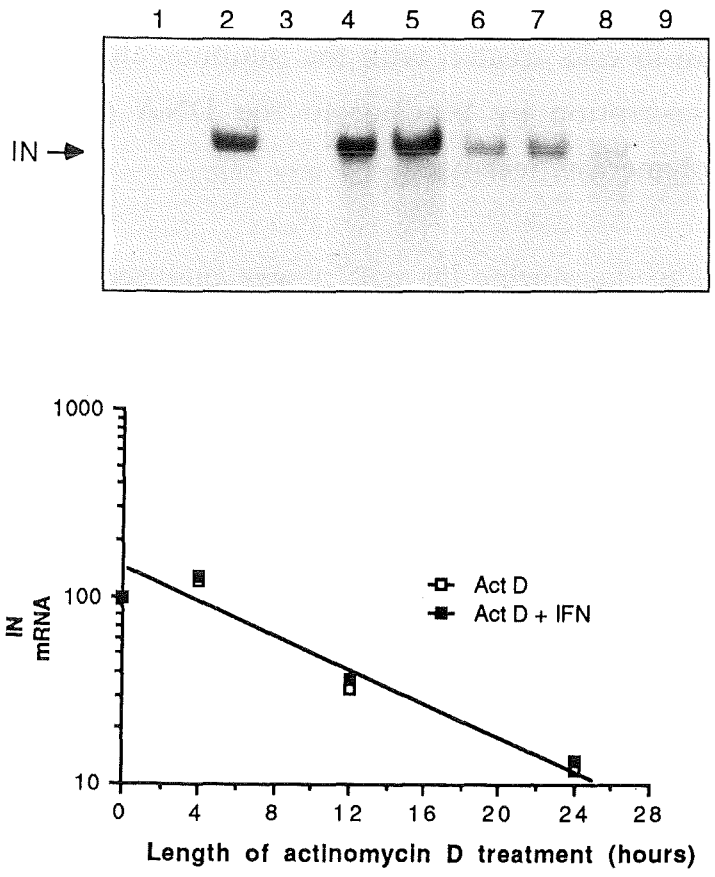


Fig. 5. IFN-γ does not prolong the half-life of the murine invariant chain RNA. Cos 10.7 clone 1 was treated with 400U/ml IFN-γ for 48 hours to achieve maximum IN expression (lanes 2, 4-9).

Lane 1 contains poly A^+ RNA from untreated cells; lane 2, poly A^+ RNA from cells treated with 400U/ml IFN-γ for 48 hours; lane 3, poly A^+ RNA from cells treated with 400U/ml IFN-γ and actinomycin D (5μg/ml) for 48 hours.

Lanes 4-9: The IFN-γ treated cells were treated further as follows: Cells were washed with PBS to remove IFN-γ, and actinomycin D (5μg/ml) was added in all cases.

In addition, 400U/ml IFN-γ was added in lanes 5, 7 and 9.

Lanes 4 and 5, poly A^+ RNA prepared 4 hours after actinomycin D addition; lanes 6 and 7, 12 hours after actinomycin D addition; lanes 8 and 9, 24 hours after actinomycin D addition.

RNA was separated by agarose gel electrophoresis under denaturing conditions, transferred to nitrocellulose and probed with the 3.9kb Hind III murine IN specific probe. The Northern hybridization was visualised by autoradiography and the intensity of the bands was determined by densitometric scanning. The values obtained from RNA obtained after actinomycin D treatment were calculated as a percentage of the maximum value and a graph was plotted of the log of these values against the length of actinomycin D treatment. A straight line was drawn through the points to determine the half-life of the murine invariant chain mRNA.

II. CHARACTERIZATION OF THE MURINE INVARIANT CHAIN GENE

Location of the sequences in the murine IN gene which respond to IFN-γ treatment required the analysis of its genomic clone. A map of cos 10.7 which contains the murine IN gene is shown in Fig. 1. The position of the IN gene is indicated; it has been located to a 10kb Eco RI fragment (Yamamoto et al., 1985b). This fragment was subcloned into the vector pUC9 (Vieira and Messing, 1982) by H.J. Rahmsdorf (Rahmsdorf et al., 1986). A partial restriction map of this subclone and one of the overlapping 13kb Pst I-Hind III subclone is also shown in Fig. 1. Analysis was concentrated on these two fragments with the aim of locating the start of transcription of the murine IN gene and the 5′ flanking sequences where the regulatory sequences of a gene are normally located.

a. The orientation of the murine invariant chain gene within cos 10.7.

The first step in the identification of the 5′ flanking region of the murine IN gene was to determine the orientation of transcription of the gene within cos 10.7. This was carried out by investigating which of the DNA strands hybridizes to murine IN RNA: The 10kb Eco RI fragment was cloned in both orientations into the multiple cloning site (MCS) of the vector pSP64 (Melton et al., 1984). This plasmid contains the bacteriophage SP6 promoter (immediately upstream of the MCS) which directs transcription of RNA with high efficiency. An *in vitro* transcription was carried out with these constructs in the presence of radioactively labelled UTP. The synthesized RNAs were hybridized, in a Northern blot hybridization, to RNA from cos 10.7 clone 2 transfectants either untreated or treated with IFN-γ for 72 hours. Hybridization occurred as expected with one RNA probe only, namely that synthesized from the DNA cloned in the antisense orientation (Fig. 6). From this analysis the direction of transcription of the IN gene was deduced to be from left to right on Fig. 1.

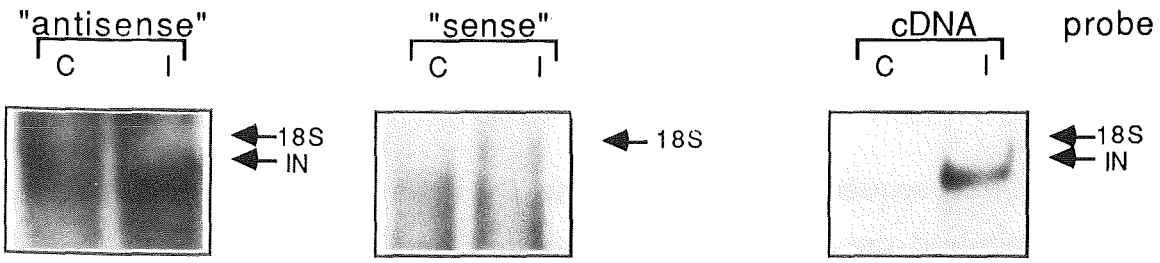


Fig. 6. Determination of the orientation of transcription of the murine invariant chain gene in cos 10.7.
The 10kb Eco RI fragment was cloned in both orientations into the MCS of the vector pSP64 (Melton et al., 1984). An *in vitro* transcription was carried out from both orientations in the presence of α ^{32}P UTP to produce "sense" and "antisense" RNA probes. These probes were hybridized to RNA from untreated cos 10.7 clone 2 transfectants (lanes marked C) or RNA from cells treated with 100U/ml IFN-γ for 72 hours (lanes marked I), which were fixed to nitrocellulose after separation by agarose gel electrophoresis. As a control for the size of the murine IN RNA, the same RNAs were also hybridized to the murine IN cDNA (Singer et al., 1984).

b. The start of transcription of the murine invariant chain gene is located within a 3kb Pvu II fragment of cos 10.7.

To determine the start of transcription in cos 10.7, I analysed the distribution of IN coding sequences within the two subclones, the 10kb Eco RI and the 13kb Pst I-Hind III clones. First, the five Hind III fragments from the 10kb Eco RI subclone were investigated. Each one was isolated, nick translated and hybridized against RNA from cos 10.7 clone 2 cells untreated or treated with IFN-γ (100U/ml) for 72 hours. No hybridization was obtained with RNA from untreated cells but all fragments except the most 3′ fragment hybridized to RNA from IFN-γ treated cells which showed the correct size for the IN RNA (Fig. 7). This indicates that at least four of the five Hind III fragments contain coding sequences for the murine IN gene.

It is possible that the 5′ end of the gene is located further upstream since the most 5′ fragment of this clone hybridized to IN mRNA. Therefore the overlapping 13kb Pst I-Hind III fragment was also analysed for the presence of IN coding sequences. The four Pvu II fragments were hybridized in the same way as the Hind III fragments of the 10kb Eco RI fragment. Two of these fragments, one

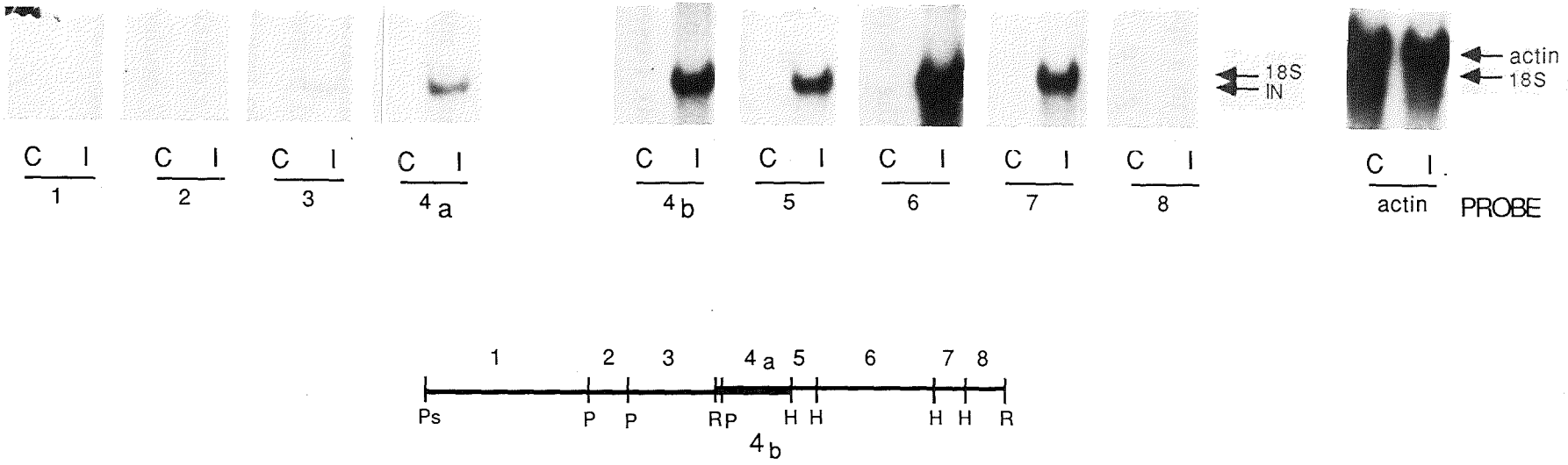


Fig. 7. The distribution of the coding sequences of the murine invariant chain gene in the adjacent restriction fragments of the 13kb and the 10kb subclones.
The DNA fragments 1-8 were isolated from the Pst I-Hind III and Eco RI subclones of cos 10.7 (shown here together as one contiguous fragment) and were radioactively labelled by nick translation. They were then probed against RNA from untreated cos 10.7 clone 2 transfectants (lanes marked C) or RNA from the same cells treated with 100U/ml IFN-γ for 72 hours (lanes marked I), which was fixed to nitrocellulose after separation by agarose gel electrophoresis under denaturing conditions. The fragments in the left panel were tested independently of those in the right panel. This resulted in a difference in intensity of the hybridization signals due to the different specific activities of the probes. As a control that equal amounts of RNA from untreated and IFN-γ cells were used, the RNAs were hybridized to a probe from the human α-actin gene (Minty et al., 1981).

of which overlaps the most 5′ Eco RI fragment, hybridized to IN mRNA. The distribution of the DNA fragments which hybridized to IN specific RNA is shown in Fig. 7. Hybridization of the same RNAs with the probe for the actin gene whose expression is not influenced by IFN-γ treatment, is also shown as a control that equal amounts of RNA from untreated and IFN-γ treated cells were used. The most extreme 5′ fragment of DNA which hybridizes to IN RNA, is a 3kb Pvu II fragment. This probably contains the start of transcription of the murine IN gene. The usual way to determine the start of transcription is by S1 nuclease protection analysis where it can be mapped with respect to a specific restriction enzyme site located within the first exon of the gene. Since information on restriction sites in the first exon was not available this was not yet possible.

c. Sequencing the murine invariant chain mRNA provides the required information for S1 nuclease protection analysis.

The sequence of the murine IN cDNA has been published (Singer et al., 1984). However the isolated and described cDNA clone was not complete. It was found to be lacking the sequences corresponding to the amino-terminal 50 amino acids and the 5′ untranslated sequences of the IN gene (Singer et al., 1984). I used the sequence information available to synthesize an oligonucleotide of 18 nucleotides corresponding to sequences close to the 5′ end of the cDNA clone isolated by Singer (positions +244 to +262). This oligonucleotide was used as a primer to sequence the murine IN RNA by the chain termination method (Sanger et al., 1977).

First, a primer extension reaction was carried out to measure the distance from the primer to the 5′ end of the IN mRNA. Using the 5′ end-labelled primer and the enzyme reverse transcriptase, a DNA strand complementary to murine IN RNA was synthesized and the reaction products were separated on a denaturing polyacrylamide gel. A major extension product of approximately 247 nucleotides was obtained. This is shown as a band of 265 nucleotides (with primer) in Fig. 8

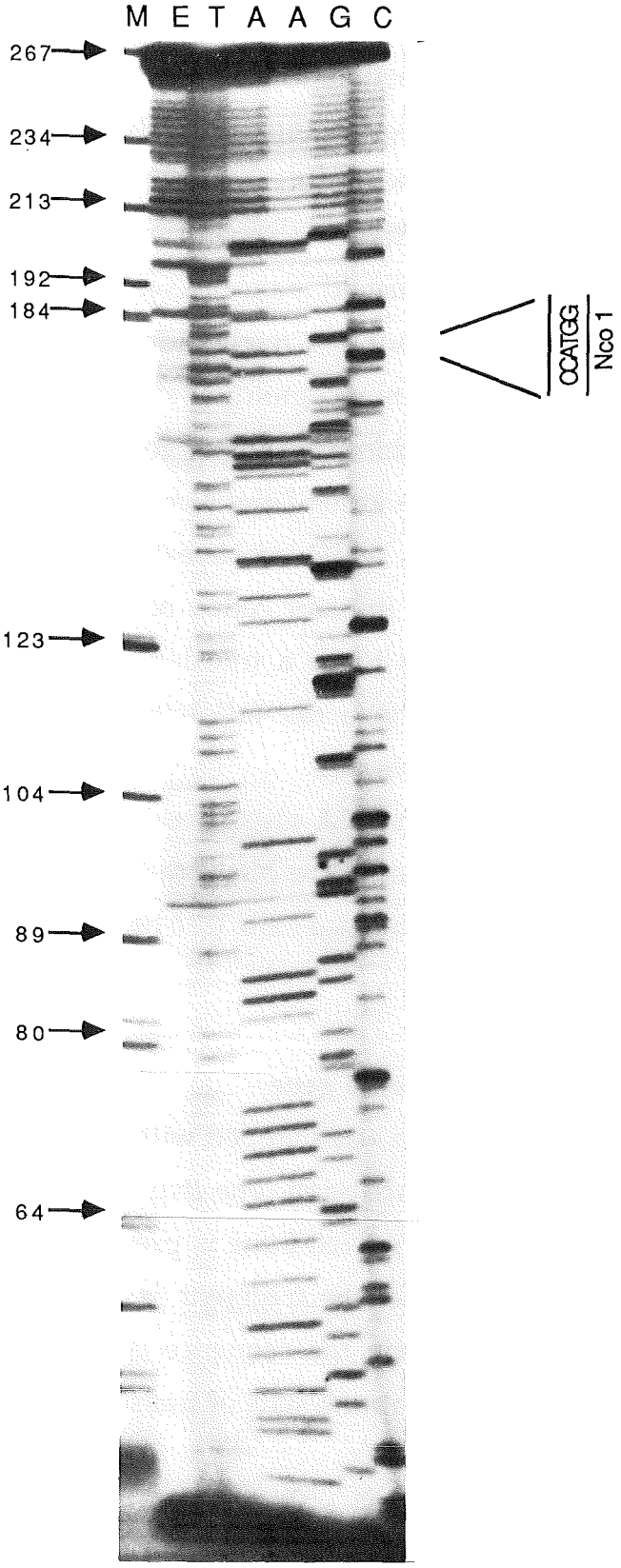


Fig. 8. Primer extension analysis and sequencing of RNA from the transfected murine invariant chain clone provides the information necessary to locate the start of transcription.
Primer extension analysis of RNA from cos 10.7 clone 1 transfectants treated with 100U/ml IFN-γ for 72 hours was carried out using an end-labelled primer from the second exon of the murine IN gene (methods IV.4), lane marked "E". Sequencing of the RNA was carried out by including dideoxynucleotide triphosphates in the reaction, the lane marked "T" contains ddTTP and so on. Two different concentrations of ddATP were used (0.7mM and 1.2mM) but no difference is seen between them. M = marker, pBR322 cut with Hae III. The sequence and position of the restriction site Nco I, which contains the ATG translation initiator codon, is shown.

in the lane marked "E". There are several minor bands also present in the same lane, which are probably due to premature halts on the RNA by the reverse transcriptase. The extension product was sequenced by including dideoxy-nucleotide triphosphates in the primer extension reaction. The sequence of about 180 nucleotides of RNA could be read from the sequencing ladder (Fig. 8). By comparison with the human IN DNA sequence, (Kudo et al., 1985), the RNA sequence was identified as being encoded by the first and second exons of the murine IN gene. The most important information gleaned from the sequence was the presence of a unique Nco I restriction site about 160 nucleotides upstream from the primer. An Nco I site incorporates within its recognitition site an "ATG" codon which can be used as a translation start codon and which codes for the amino acid methionine. Comparison of the sequence around the Nco I site of the murine IN gene with the human IN amino acid sequence revealed that the "ATG" of the Nco I site corresponded to the second initiator codon of the human gene. The "ATG" of the Nco I site was then taken to be a translation initiator codon of the murine IN gene. The thirteen amino acids following the ATG initiator codons are identical in both genes (not shown).
The primer extension analysis of the murine IN mRNA also revealed that the start of transcription is about 90 nucleotides upstream of the Nco I restriction site. This was mapped more exactly by S1 analysis using the Nco I site (see below).

d. DNA sequence analysis of the 5′ end of the murine IN gene

The Nco I site of the first exon of the murine IN gene was mapped within the 3kb Pvu II fragment thought to contain the start of transcription. The Pvu II fragment was cloned into pSP65, yielding cos 10.7/6. Only one Nco I site is present in this fragment of DNA and it lies 1.3kb from the 5′ end of the fragment or 1.7kb from the 3′ end of the fragment. It was used a) to obtain the sequence of the DNA directly upstream from it and b) in S1 analysis, to map the start of transcription on the genomic clone (see below).

The DNA subclone, cos 10.7/6, was sequenced a) from the Nco I site in the

direction of the 5′ end and b) from position -347 of the murine IN gene (see methods II.13) by the chemical cleavage method of Maxam and Gilbert (1980). The DNA sequence was also obtained by the chain termination method (Sanger et al., 1977) using a primer which hybridizes to the first exon of the murine IN gene from positions +110 to +126. The DNA sequence of the murine IN gene from positions -347 to +161 is shown in Fig. 9. The sequence revealed the following interesting features:

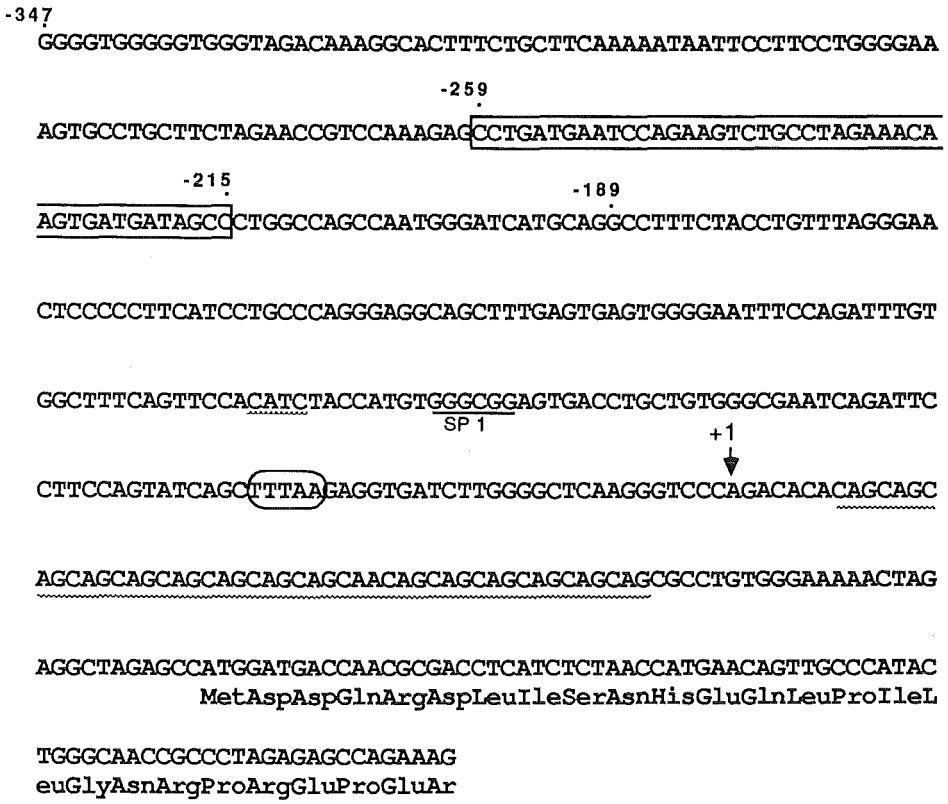
1. At positions -32 to -28, the sequence TTTAA was found, identical to that of the human IN gene (Kudo et al., 1985; O'Sullivan et al., 1986) which shows homology to the "TATA" consensus sequence important for specific initiation of transcription (Breathnach and Chambon, 1981). The sequence CATCT, located at positions -92 to -88, is homologous to the "CCAAT" consensus sequence (Breathnach and Chambon, 1981).

2. A consensus Sp1 binding site (GGGCGG; Dynan and Tjian, 1983; Gidoni et al., 1984) was found between the CCAAT and the TATA boxes.

3. The start of transcription, deduced from the primer extension analysis and mapped also by S1 nuclease protection analysis (see below) is indicated, +1 indicates the major start site. There are 85 nucleotides of 5′ untranslated sequences upstream of a unique ATG codon. This is in contrast to the situation in the human IN gene where two in-frame initiator ATG codons are present. Both initiator codons have been shown to be functional although translation of the human IN gene initiates predominantly at the second ATG (Strubin et al., 1986a). It is the latter which is homologous to the initiator codon of the murine IN gene (Fig. 9b).

5. Also at variance with the human IN gene is the presence of a trinucleotide repeat "CAG" in the 5′ untranslated region of the murine IN gene (Fig. 9). This triplet is repeated 16 times with one base pair change. This repeat sequence has been identified in other genes of a variety of organisms, including several bovine genes, the murine interleukin-2 gene (NIH Genetic Sequence Data Bank and D. Dobbelaere, pers. comm.) and some homeotic genes of Drosophila (Wharton et al., 1985). The significance of its presence in these genes is as yet unclear.

A



B

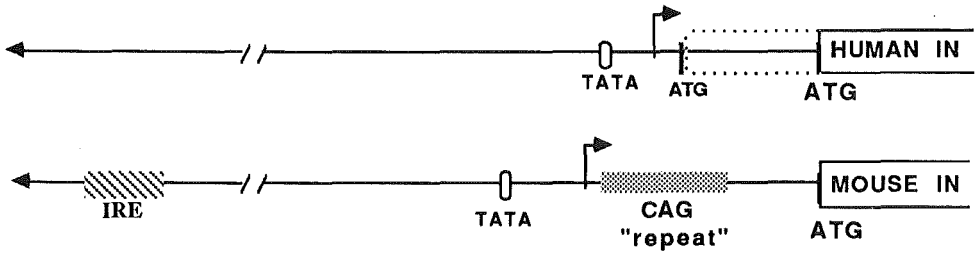


Fig. 9. DNA sequence of the 5´ flanking region and the first exon of the murine invariant chain gene.

A. The sequence of the 5´ end of the cloned murine IN gene on cos 10.7 from positions -347 to +109 was determined by the methods of both Maxam and Gilbert (1980) and Sanger et al. (1977) as modified by Chen and Seeburg (1985).The sequence of the first exon was also obtained by sequencing the IN RNA (see results section c and Fig. 8).The amino acid sequence of the coding region is given in the 3-letter code below the DNA sequence. +1 indicates the major start of transcription. The positions of the "TATA" box, "CAAT" box and Sp1 binding site, are indicated. The CAG repeat sequence is underlined. The square box encloses the IRE sequence which was determined by analysis of deletion mutants (results, section III).

B.Comparison of the structure of the 5´ regions of the human and murine IN genes. They are aligned at the start of translation (in the case of the human gene, at the second initiator codon). Arrows indicate the start of transcription; the dotted box indicates the alternative translated region of the human gene. The positions of the CAG repeat and the IRE are indicated.

6. The 5′ flanking sequences of the murine and human IN genes are 74% homologous.

While this work was in progress, the murine IN gene was sequenced from position -250 to its poly A site by W. Lauer (Koch et al., 1987). Both sequences are identical in the overlapping region.

e. S1 mapping of the start of transcription of the murine invariant chain gene within the 3kb Pvu II fragment.

The start of transcription of the murine IN gene within the 3kb Pvu II fragment was mapped as follows: The 1.3kb Pvu II-Nco I fragment, 5′ end-labelled at the Nco I site, was used as a probe. It was hybridized to RNA from cos 10.7 clone 1 transfected GM637 cells either untreated (control) or treated with IFN-γ (100U/ml) for 72 hours. The hybrids were digested with S1 nuclease which cuts single stranded nucleic acids (Berk and Sharp, 1977). The fragment of DNA protected by the RNA was analysed by denaturing polyacrylamide gel electrophoresis. A major protected fragment, 88 nucleotides in length and several larger minor fragments were seen with RNA from IFN-γ treated cells only (Fig. 10, lanes 1, 2, 2a). The analysis was also carried out on RNAs from the spleenocytes of three murine strains. This was done to compare the initiation of the IN RNA from the transfected gene with that in the animal. The initiation of IN RNA in the murine lymphoma cell line CH1.1 which expresses the IN gene (Rahmsdorf et al., 1983, 1984; Koch, S. et al., 1984) was also analysed.

The results of this analysis were interesting for several reasons: most importantly, it shows that RNA initiation from the transfected murine IN gene is correct. The pattern of S1 nuclease protected DNA fragments in the transfected gene is identical to that obtained with RNA from AKR mice from which the cosmid clone was derived, (Fig. 10, compare lanes 2 and 5). RNA initiation from IN genes in C57Bl mice and the cell line CH1.1 is also identical, (Fig. 10, lanes 3 and 7). In Balb/c mice, the situation is different. The S1 protected fragments obtained with RNA from Balb/c mice are smaller than those obtained with all other RNAs tested, being only about 50 to 60 nucleotides long (Fig. 10, lane 6).

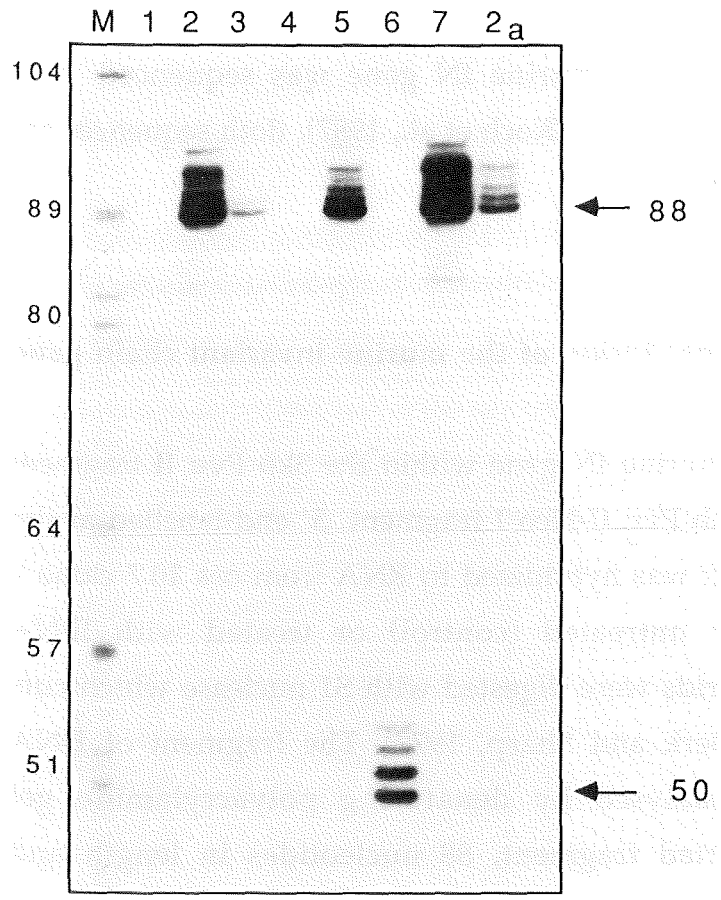
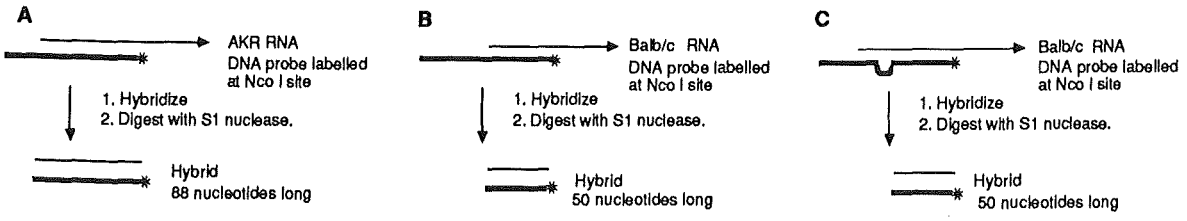


Fig. 10. S1 nuclease analysis of murine invariant chain RNA.
3μg poly A^+ RNA was analysed using the Pvu II-Nco I fragment labelled at the Nco I site as a specific probe. Lane 1, RNA from untreated cos 10.7 clone 1 transfectants; lane 2, RNA from the same cells treated with 100U/ml IFN-γ for 72 hours; lane 2a, as lane 2 but only 1/5 amount of radioactivity was loaded onto this lane; lane 3, spleenocyte RNA from the C57Bl strain; lane 4, tRNA control; lane 5, spleenocyte RNA from the AKR strain; lane 6, spleenocyte RNA from the Balb/c strain; lane 7, RNA from the CH1.1 cell line. M = marker, pBR322 cut with Hae III. The numbers, 88 and 50 are the size of the major protected fragments in nucleotides.
A, B, and C below are schematic representations of the hybridization of the RNAs to the DNA probe. A, for all IN RNAs except Balb/c IN RNA; B and C, two possiblities for the hybridization of Balb/c IN RNA to the probe.



This is not due to RNA degradation since several RNA preparations gave the same result. There are two possible explanations for the shorter length of the Balb/c S1 protected fragment: (1) the 5′ untranslated region of the Balb/c murine IN gene is shorter than that of AKR or C57Bl mice, (2) the 5′ untranslated region of the Balb/c murine IN gene differs from that of AKR or C57Bl mice about 50

nucleotides upstream of the Nco I site (ATG initiator codon). This would cause imperfect hybridization to the probe from the cloned AKR IN gene resulting in an incorrect interpretation of the S1 protection analysis. I have distinguished between these possibilities by carrying out a primer extension analysis of Balb/c RNA and comparing it with that obtained from the transfected gene, AKR spleenocytes and the cell line CH1.1.

A primer corresponding to sequences downstream of the ATG initiator codon from positions +110 to +126 was used. An extension product of 130 nucleotides was obtained with RNA from the transfected gene, the endogenous genes in AKR spleenocytes and the cell line CH1.1 as compared with 124 nucleotides with RNA from Balb/c mice (Fig. 11). This is a difference of 6 nucleotides as compared to a difference of 30 to 35 nucleotides in the S1 analysis. It seems probable therefore, that there is just a small difference in the sequence of the 5′ untranslated region between the Balb/c IN RNA and the IN RNA from the other murine strains. This may be a difference in the "CAG" repeat; for a final proof however, the Balb/c IN gene would have to be isolated and sequenced.

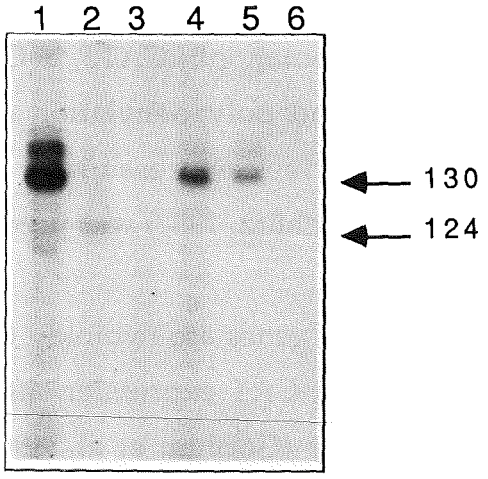


Fig. 11. Primer extension analysis of different murine invariant chain mRNAs.
3µg poly A^+ RNA were analysed with an end-labelled oligonucleotide primer from the first exon of the transfected murine IN gene (methods IV,4). Lane 1, RNA from the cell line CH1.1; lane 2, spleenocyte RNA from the Balb/c strain; lane 3, tRNA control; lane 4, spleenocyte RNA from the AKR strain; lane 5, RNA from cos 10.7 clone 1 transfectants treated with 100U/ml IFN-γ for 72 hours; lane 6, RNA from untreated cos 10.7 clone 1 transfectants.

III. FUNCTIONAL ANALYSIS OF THE 5′ FLANKING REGION OF THE MURINE INVARIANT CHAIN GENE

The transcriptional activity of a gene is regulated by the interaction of cis-acting DNA elements with trans-acting factors (Ptashne, 1986). Having determined that IFN-γ activates the transcription of the murine IN gene, I analysed the 5′ flanking region of this gene to locate the cis-acting sequences which confer IFN-γ inducibility. The regulatory properties of a DNA fragment can be demonstrated, if after transfection into eukaryotic cells, this fragment can be shown to influence the expression of an indicator gene in the presence of the relevant inducer. Viral or prokaryotic genes are usually used as indicator genes; for example, the coding region of the thymidine kinase gene of Herpes simplex virus (Chandler et al., 1983, Hynes et al., 1983) or the bacterial gene coding for chloramphenicol acetyl transferase (CAT; Gorman et al., 1982).

The coding region of the bacterial CAT gene was used in the analysis of the 5′ flanking sequences of the murine IN gene. The bacterial sequences do not contain promoter sequences and can only be expressed in eukaryotic cells with the help of a fused eukaryotic promoter. pBLCAT3, a CAT expression vector which contains the small t intron and polyadenylation signals from the SV40 virus which are necessary for correct expression of the CAT protein in eukaryotes (Fig. 12 and Luckow and Schütz, 1987) was used in the analysis.

The advantages of hybrid CAT fusion genes are twofold: there is no endogenous CAT activity in eukaryotic cells, and CAT expression after transfection can be tested in a rapid and sensitive assay (Gorman et al., 1982). The enzyme CAT transfers acetyl groups from a donor molecule, acetyl coenzyme A, to an acceptor molecule, chloramphenicol. The unacetylated and acetylated forms of chloramphenicol can be separated by TLC chromatography. If the chloramphenicol is radioactively labelled, the reaction products can be visualised by autoradiography. When the cell extracts are incubated with an excess of chloramphenicol and acetyl coenzyme A, the amount of acetylated

chloramphenicol is a direct measurement of the amount of CAT protein present.

a. The 5′ flanking sequences of the invariant chain gene confer inducibility by interferon-γ to an indicator gene.

The murine IN 5′ flanking sequences from positions -1200 to +85 were cloned into pBLCAT3 (Fig. 12). The resulting construct was named pIN-1200/+85CAT. This construct was transiently transfected into GM637 cells by the DEAE-dextran procedure. Cells were harvested after 40 hours treatment with IFN-γ (400U/ml) and the cell extracts were assayed for CAT activity. The CAT activity in cells transfected with the vector pBLCAT3 was around background level (background level is the level of acetylated chloramphenicol measured in a CAT assay in the absence of CAT protein, usually 0.1-0.3% of unacetylated chloramphenicol). Cells transfected with pIN-1200/+85CAT displayed a basal level of CAT activity 3- to 10-fold above background level. This basal level of activity was increased

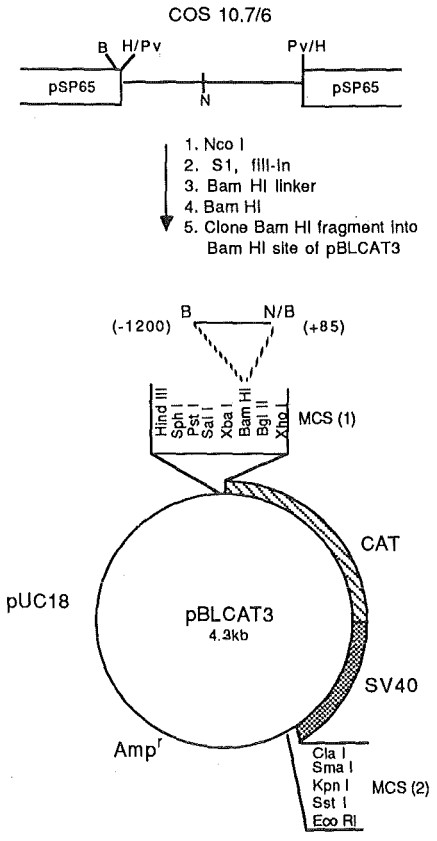


Fig. 12. Cloning of the 5′ flanking sequences of the murine invariant chain gene into pBLCAT3.
The Bam HI-Nco I fragment from cos 10.7/6 (methods II.13) was cloned into the pBLCAT3 vector of Luckow and Schütz (1987), resulting in pIN-1200/+85CAT. The restriction sites are: B = Bam HI, H = Hind III, N = Nco I, Pv = Pvu II. MCS = multiple cloning site. The construction of all other IN-CAT constructs is described in methods II.13.

around 8-fold by IFN-γ treatment (Table 2). This demonstrates that the sequences required for the basal level of expression of the IN gene and those which confer inducibility of the gene by IFN-γ are present in the clone pIN-1200/+85CAT.

b. Deletion analysis of the 5′ flanking sequences of the murine invariant chain gene delimits a 44bp long interferon-γ response element.

Delimitation of the cis-acting sequences which confer IFN-γ inducibility to the murine IN gene was carried out by deletion analysis of the IN sequences in clone pIN-1200/+85CAT. A diagram of the resulting CAT constructs and their inducibility by IFN-γ is given in Fig. 13.

Deletions from the 5′ end of the IN flanking sequences were carried out by Bal 31 digestion from the Hind III restriction site of pBLCAT3, (methods II.13). Deletion to positions -261 or -259 (constructs pIN-261/+85CAT and pIN-259/+85CAT) did not result in any significant loss of basal activity of the IN promoter or IFN-γ inducibility (Figs. 13 and 14). The CAT activity from these deletion mutants was 7-12 times higher in IFN-γ treated cells than in untreated cells. The 5′ deletion mutant, pIN-254/+85CAT, is still 3-fold activated by IFN-γ, the mutant pIN-246/+85CAT, less than 2-fold. The basal activity from the deletion mutant pIN-234/+85CAT is not induced by IFN-γ. Therefore the 5′ border of the element which confers IFN-γ inducibility to the murine IN gene must lie between positions -254 and -234. The autoradiogram of a representative CAT assay of 5′ deletion mutants is shown in Fig. 14.

Delimitation of the 3′ border of the IFN-γ response element was carried out from position -189 in pIN-261/+85CAT at which the restriction enzyme Stu I cuts. A Pst I site was inserted at this position to facilitate cloning. This had no influence on the basal level or IFN-γ induced expression of this construct (Fig. 13). The activity of the construct pIN-189/+85CAT was not induced by IFN-γ but retained a basal level of activity (Fig. 15). For convenience, this is referred to as the IN promoter. Placing the sequences -261/-210 or -261/-215 in front of the IN promoter (pIN-261/-210,-189/+85CAT and pIN-261/-215,-189/+85CAT) conferred

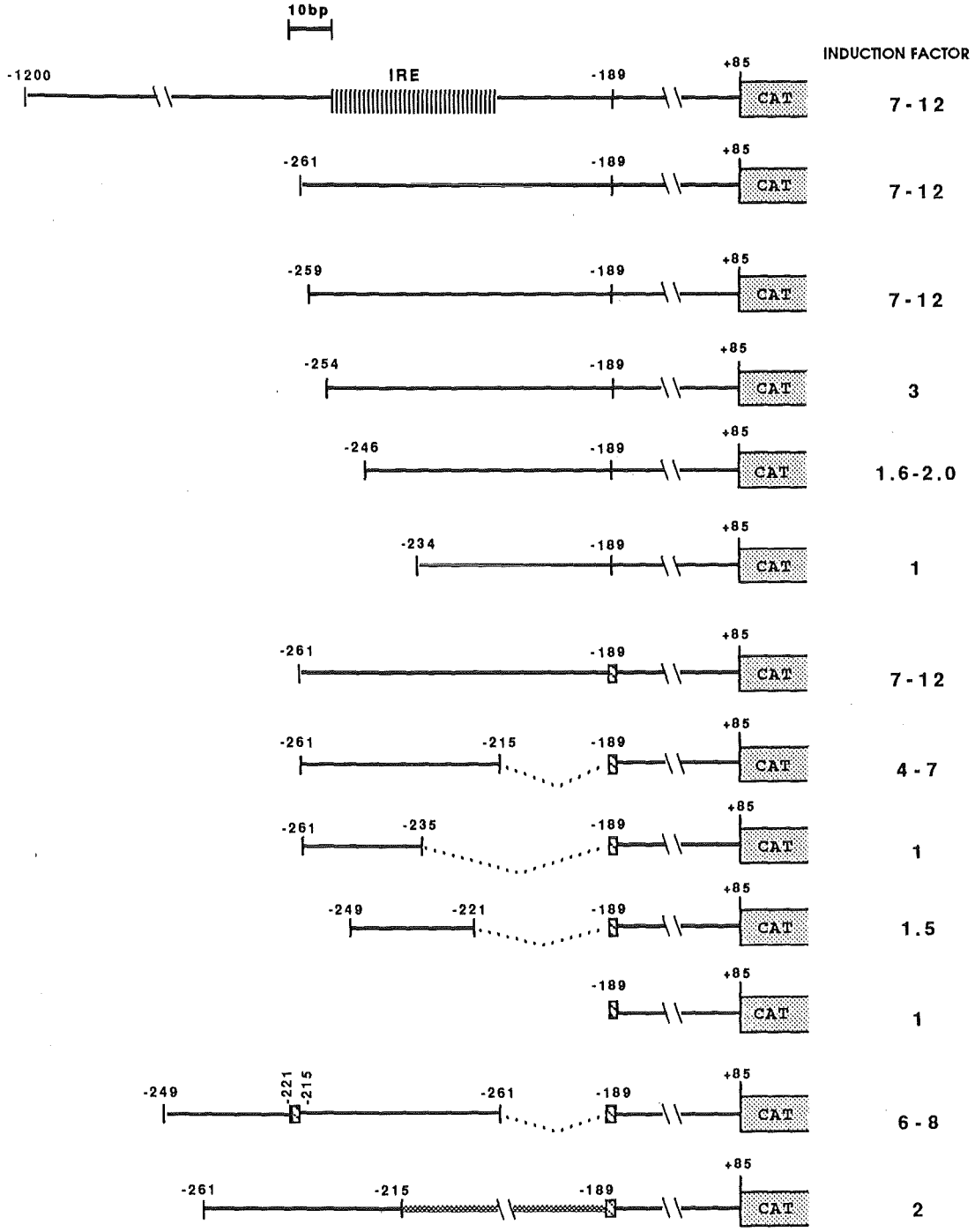


Fig. 13. Chimaeric IN-CAT deletion constructs and their response to IFN-γ (induction factors).
The structure of IN-CAT constructs is shown: Black horizontal lines represent 5′ flanking sequences of the murine IN gene. Numbers correspond to the distance in nucleotides from the start of transcription. The CAT gene is represented by a dot-filled rectangle; Pst I linker by a rectangle with diagonal stripes and the IRE sequences by vertical stripes. The thick dotted line in the last construct represents the 326bp Bam HI-Bgl II fragment from the 3′ noncoding region of the human collagenase gene (Angel et al., 1987). Each construct is named according to the location of its deletion ends, for example, the largest construct is pIN-1200/+85CAT.The increase in expression (induction factor) of each construct following IFN-γ treatment after transient transfection into GM637 cells is shown along the right hand side. The values are the mean values of at least three different experiments.

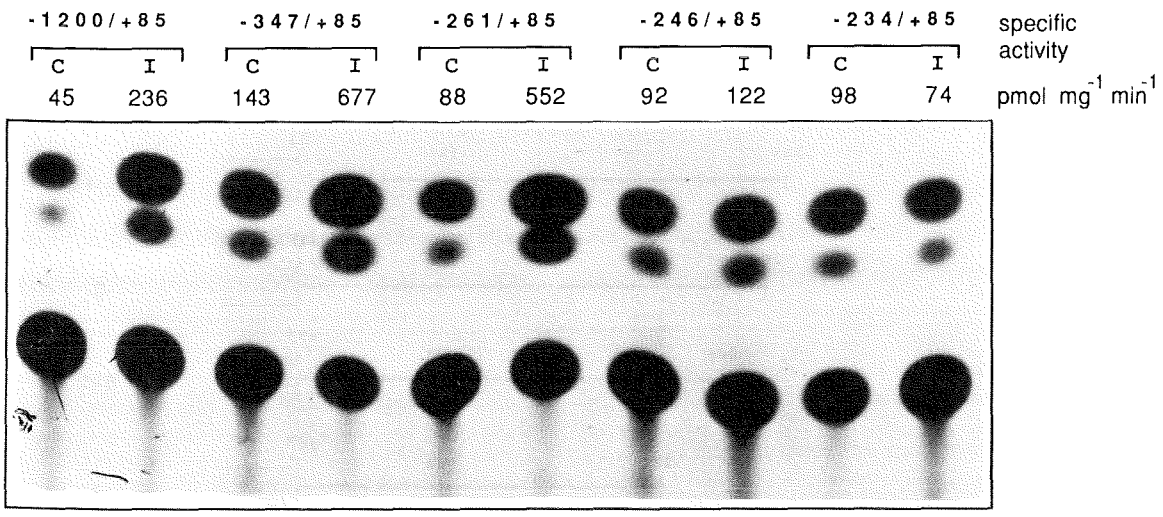


Fig. 14. Analysis of IFN-γ inducibility of 5′ deletion mutants.
10μg of each CAT construct were transfected transiently into GM637 cells by the DEAE-dextran method and CAT activity was analysed: C, CAT activity in untreated cells; I, CAT activity in IFN-γ treated cells (400U/ml, 40 hours). Specific CAT activity is in pmol $mg^{-1}min^{-1}$.

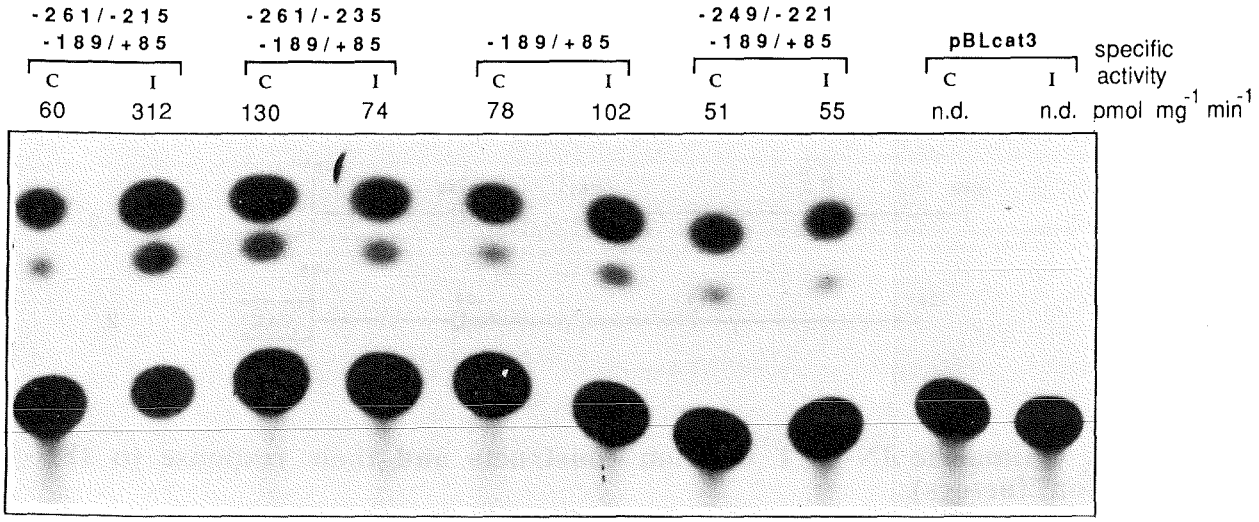


Fig. 15. Analysis of IFN-γ inducibility of 3′ deletion mutants.
10μg of each CAT construct were transfected transiently into GM637 cells by the DEAE-dextran method and CAT activity was analysed: C, activity in untreated cells; I, activity in IFN-γ treated cells (400U/ml,40 hours). Specific activity is in pmol $mg^{-1}min^{-1}$. n.d. = not determined (activity was at background level).

IFN-γ inducibility; the induction was 4-7-fold as compared to 7-12-fold for the original construct, pIN-261/+85CAT. The difference may be due to position effects. The activity of the construct pIN-261/-235,-189/+85CAT was not IFN-γ inducible (Fig. 15). Therefore the 3′ border of the element conferring IFN-γ inducibility lies between positions -235 and -215. Thus by deletion mutant analysis, the minimum IFN-γ response element (IRE) has been located between positions -254 and -215 of the murine IN gene. For full induction by IFN-γ the sequences -259 to -215 are required. The specific CAT activities of the IN-CAT constructs following transfection into GM637 cells in the presence and absence of IFN-γ, described in this section are given in Table 2.

Table 2: CAT activities of deletion mutants of the 5′ control region of the murine invariant chain gene.

CONSTRUCT	CONTROL	INTERFERON-γ	INDUCTION FACTOR	NUMBER OF EXPERIMENTS
pBLCAT3	85 ± 15	64 ± 12	0.8	5
-1200/+85	160 ± 27	1372 ± 183	8 - 11	5
-347/+85	189 ± 52	1650 ± 485	8 - 11	3
-261/+85	158 ± 17	1658 ± 299	8 - 11	12
-254/+85	149 ± 41	440 ± 190	3	2
-246/+85	143 ± 35	255 ± 87	2	2
-234/+85	226 ± 36	268 ± 81	1	6
-261/-215 -189/+85	91 ± 16	426 ± 53	4 - 7	4
-261/-235 -189/+85	230 ± 98	190 ± 141	1	2
-189/+85	244 ± 93	287 ± 89	1	4

The table gives the specific activities of IN-CAT constructs in the presence or absence of IFN-γ after transient transfection into GM637 cells by the DEAE-dextran method. Specific activities (with standard errors) are given in pmol $mg^{-1}min^{-1}$.

I compared the sequence of the functionally defined IRE with published sequences of IFN-γ responsive genes including the class II genes of the MHC. Two research groups had already published interesting compilations of sequences: (1) Basta et al. (1986) compared the 5′ flanking sequences of several genes whose expression is known to be induced by IFN-γ. They have defined a 9-mer sequence as being common to all genes investigated and suggested its involvement in the IFN-γ regulation of these genes. (2) O'Sullivan et al. (1986) compared the 5′ flanking sequences of class II genes and genes coordinately regulated with them. They found two regions of homology within the different genes: a 15-mer and an 8-mer (different from the 9-mer described by Basta et al., 1987) and proposed that these sequences are important in the regulation of the genes that contain them. Both groups compiled consensus sequences from the most homologous regions of DNA between the different genes that they compared (Table 3).

Sequences homologous to both the 9-mer and the 15-mer consensus sequences were found in the 5′ flanking region of the murine IN gene each with only one deviation from the consensus sequences (Table 3). These sequences are located in the region defined by functional analysis to confer IFN-γ inducibility to the murine IN gene. Sequences homologous to the 8-mer consensus sequence were not found. In the murine IN gene, the 9-mer and the 15-mer sequences lie contiguously between positions -246 and -223 (Table 3). In the human IN gene, the arrangement is identical. In fact, 38 out of 44 nucleotides from the region of the murine gene containing the functionally defined IRE, are identical between the human and murine IN genes.

An attempt was made to reduce the length of the functionally defined IFN-γ (-254/-215) response sequences of the murine IN gene. An oligonucleotide corresponding to positions -249 to -221 was synthesized and cloned in front of the IN promoter (pIN-249/-221,-189/+85CAT). This comprises essentially just the two consensus sequences mentioned above, surrounding them on both sides by

two base pairs (bp). The CAT activity from this construct was not significantly inducible by IFN-γ (Fig. 15).

So, although the sequences -249/-221 are necessary, they are not sufficient for full induction of expression of the IN gene by IFN-γ. This was already indicated by the 5′ deletion analysis in which the activity from the construct pIN-254/+85CAT was shown to be only 3-fold inducible by IFN-γ. Therefore no attempt was made to decrease the length of the IFN-γ response element of the IN gene any further.

Table 3: Comparison of invariant chain sequences with consensus sequences from IFN-γ inducible genes.

sequence	9-mer (1)	15-mer (2)	8-mer (2)	Reference
consensus	AGAAGN$^{A}_{C}$AG	CC$^{T}_{C}$AGN$^{A}_{G}$ACAGATGA	CTGATTGG	(1) Basta et al., 1987 (2) O'Sullivan et al., 1986
mouse IN	$^{-246}$AGAAGTCTG^{-238}	$^{-237}$CCTAGAAACAAGTGA^{-223}	———	This work
human IN	$^{-418}$AGAAGAGGA^{-410} $^{-236}$AAAGGCCTG^{-228}	$^{-227}$CCCAGAAACAAGTGA^{-213}	$^{-178}$TTGCTTGG^{-171}	O'Sullivan et al., 1986

Nucleotides deviating from the consensus sequences are underlined

c. S1 analysis of RNA transcribed from IN-CAT constructs confirms that RNA is correctly initiated.

I have defined the sequences which confer IFN-γ inducibility to the murine IN gene by analysis of CAT expression from deletion mutants of the 5′ flanking region of the gene. It was essential to determine whether these constructs also correctly initiate transcription since the induction following IFN-γ treatment could be a result of aberrant transcripts. Since analysis of CAT protein levels does not yield information on the transcript, S1 analysis of CAT RNA was carried out. For this reason, chimaeric IN-CAT constructs were transfected stably into GM637

analysed. S1 analysis of RNA from mass cultures of IN-CAT transfected cells either untreated or treated with IFN-γ for 48 hours (400U/ml) hybridized against an IN-CAT probe revealed that indeed transcription was correctly initiated in all constructs tested. Correct initiation of transcription results in the protection of a 367 nucleotide fragment in S1 analysis (Fig. 16). Inducibility of constructs after IFN-γ treatment mirrored the results of CAT assays (Fig. 16), that is, constructs inducible by IFN-γ in CAT assay were also shown to be inducible by S1 analysis of RNA (Fig. 16, left panel). Non-inducible constructs are shown in the right panel of Fig. 16.

Thus both by analysis of CAT activity in transient transfections and by S1 mapping of RNA in stable transfectants, the IRE of the murine IN was functionally defined.

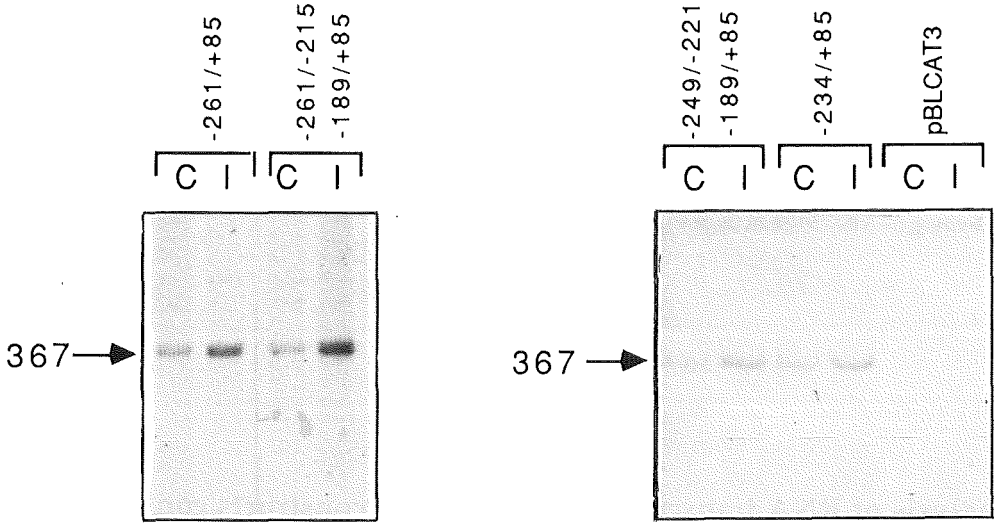


Fig. 16. S1 analysis of the transcription of IN-CAT constructs demonstrates correct initiation of transcription.
IN-CAT constructs, transfected stably into GM637 cells were analysed for the correct initiation of transcription and inducibility by IFN-γ. 20μg RNA were hybridized with an IN-CAT specific probe labelled at the Eco RI site located within the CAT gene (601bp Hind III-Eco RI fragment from pIN-234/+85CAT). Correct initiation of transcription results in a protected fragment of 367 nucleotides. C, RNA from untreated cells; I, RNA from IFN-γ treated cells (400U/ml, 48 hours).

d. Does the interferon-γ response element act like an enhancer?

Enhancers are viral or cellular DNA elements that increase the transcriptional efficiency of a gene in a manner relatively independent of their orientation and position with respect to that gene (Khoury and Gruss, 1983). They also function with a heterologous promoter and gene (Banerji et al., 1981). All enhancers studied so far, such as the SV40 and the immunoglobulin heavy chain enhancers, require for their activity the binding of trans-acting factors (Schöler and Gruss, 1984).

Many hormone response sequences have enhancer properties. For example the LTR of the mouse mammary tumour virus (MMTV) contains a glucocorticoid response element (HRE) which has enhancer properties (Ponta et al., 1985). This element is also responsive to progestins and androgens in an enhancer-like manner (Cato et al., 1987). The estrogen response element (ERE) of the Xenopus vitellogenin A2 gene also has enhancer properties (Klein-Hitpass et al., 1988). In the 2′, 5′-oligoadenylate synthetase gene there are two enhancer sequences, one of which responds to IFN-α (Cohen et al., 1988).

The IRE was tested for the following three enhancer properties:

1. Function with a heterologous promoter.

The 5′ regulatory sequences of the murine IN gene were cloned in front of the promoter of the thymidine kinase (tk) gene of herpes simplex virus, (constructs pIN-292/-73tkCAT and pIN -261/-189tkCAT; see methods II.13). The tk promoter from positions -105 to +51 is cloned in front of the CAT gene in pBLCAT2 (Luckow and Schütz, 1987). The basal level of activity of the IN-tkCAT constructs was higher than that of pBLCAT2 following transfection into GM637 cells (Fig. 17). The activity from the tk promotor of pBLCAT2 is reduced by 50% in cells treated by IFN-γ. The activity of both IN-tkCAT constructs was induced about 2-fold by IFN-γ (Fig. 17). Thus the IRE functions in front of a heterologous promoter both to mediate a basal level of transcription and IFN-γ induction of transcription.

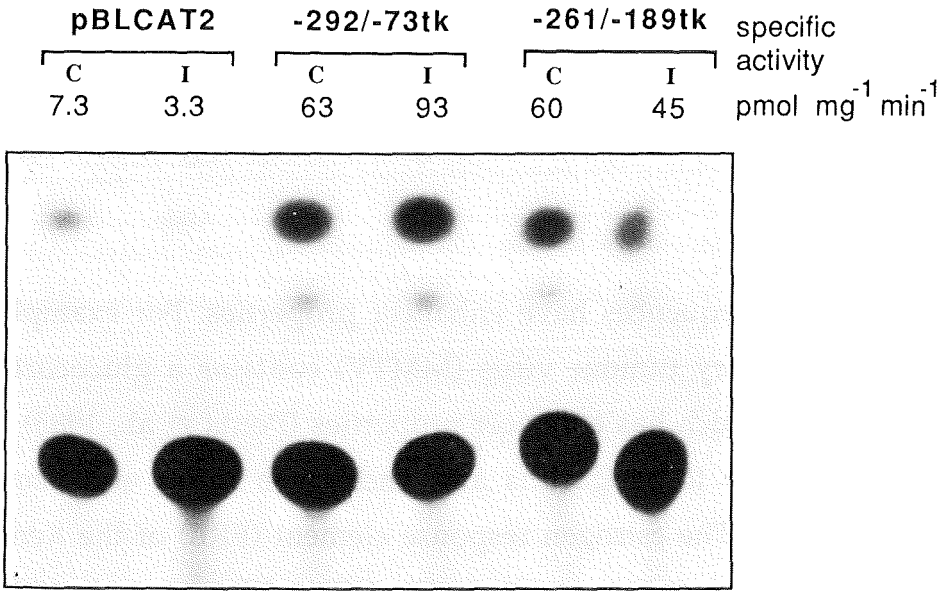


Fig. 17. The IRE functions in front of a heterologous promoter.
15μg of each tkCAT construct were transfected transiently into GM637 cells by the DEAE-dextran method and CAT activity was analysed: C, activity in untreated cells; I, IFN-γ treated cells (400U/ml,40 hours). Specific activity is in pmol $mg^{-1}min^{-1}$.

2. Function in a different position to the IN promoter

The IRE was separated from the IN promoter by the introduction of 326bp of unrelated DNA from the 3'untranslated region of the human collagenase gene (Angel et al., 1987). The activity of this construct (pIN-261/-215,■-189/+85CAT) was induced 2-fold by IFN-γ as compared with 4- to 7-fold for the original construct (Fig. 13). The IRE therefore functions weakly from a position further distant to the IN promoter than its normal position. When the sequences, -261/-215, were cloned behind the CAT gene into the Sma I site of pIN-189/+85 CAT, this construct was not significantly inducible by IFN-γ (data not shown).

3. Function in reverse orientation.

A double mutant, pIN-249/-221,-215/-261,-189/+85CAT contains the sequences -261/-215 in reverse orientation to the construct pIN-261/-215,-189/+85CAT which is 4- to 7-fold inducible by IFN-γ. It also contains the IFN-γ uninducible sequences -249/-221. The enhancement of the CAT activity of the double mutant by IFN-γ is comparable to that of the construct pIN-261/-215 (Fig. 18). Thus the IRE functions in the inverted orientation also.

This analysis demonstrates that the murine IRE fullfills the requirements of an enhancer.

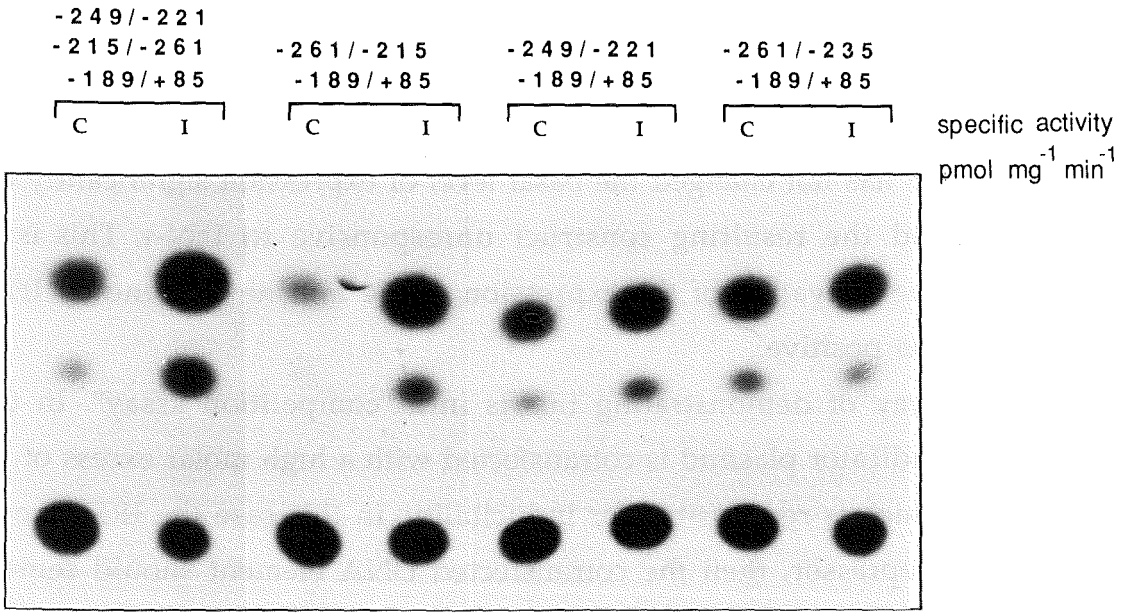


Fig. 18. The IRE functions in an inverted orientation.
10μg of each CAT construct were transfected transiently into GM637 cells by the calcium phosphate method and CAT activity was analysed: C, activity in untreated cells; I, activity in IFN-γ treated cells (400U/ml,40 hours). Specific activity is in pmol $mg^{-1}min^{-1}$.

e. Interferon-γ regulation of the murine invariant chain gene through the interferon-γ response element is mediated by a positively acting factor.

I have shown that IFN-γ induces expression the murine IN gene through the IRE by analysis of CAT RNA and protein expressed from different deletion mutants of the 5′ control region of the gene. There are at least two possible mechanisms by which this induction could occur, 1) positive regulation in which an IFN-γ induced protein binds to the IRE sequences thus activating transcription or 2) negative regulation, whereby a repressor binding to these sequences is removed by IFN-γ treatment. If the latter possibility were true, it would be expected that when the cis-acting sequences are deleted, repressor molecules would no longer be able to bind to the DNA. Then the basal level of expression of constructs lacking the IRE should be as high as the IFN-γ induced level in constructs containing the IRE. This is not true for any of the constructs of the IN gene lacking the IRE (Table 2 and Fig. 19). In each case, deletion of all or part of the IRE has not changed the basal level of expression significantly but has just rendered the resulting construct unresponsive to IFN-γ. This is an indication that the activation of the expression of the murine IN gene by IFN-γ through the IRE is positive.

A more direct way of demonstrating this is in a "competition assay". In this experiment, an indicator plasmid is cotransfected with a high molar excess of the DNA element which is responsible for inducibility, in this case the IRE. Should the IRE bind a repressor, then the cotransfected DNA element should remove the repressor from the indicator plasmid and the non-induced level of activity of the indicator plasmid should increase. When, however, IFN-γ treatment leads to the appearance of an activating molecule, the cotransfected DNA element would bind this protein and the indicator plasmid should remain unresponsive to IFN-γ. The experiment was carried out as follows: The construct pIN-261/+85CAT (which contains a functional IRE) was transfected transiently into GM637 cells with an excess of IRE sequences cloned in pSP65 (pIN-261/-215SP65 = pIRE-SP65). pSP65 plasmid DNA (Melton et al., 1984) was used as an unspecific competitor so that an identical amount of DNA was always transfected in the same experiment.

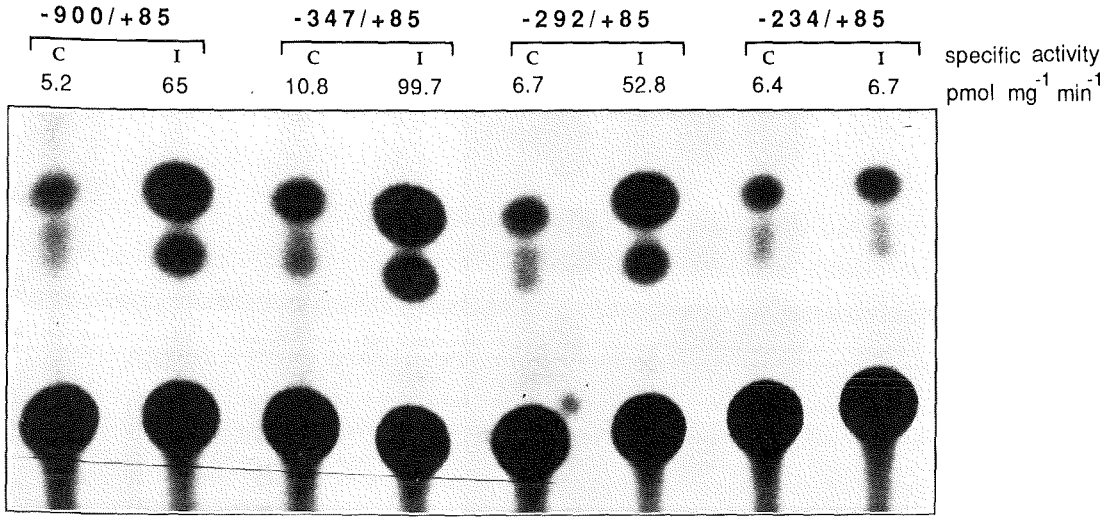


Fig. 19. The basal level of activity does not increase upon deletion of part of the IRE.
10μg of each CAT construct were transfected transiently into GM637 cells by the DEAE-dextran method and CAT activity was analysed: C, activity in untreated cells; I, activity in IFN-γ treated cells (400U/ml,40 hours). Specific activity is in pmol $mg^{-1}min^{-1}$.

In one experiment, 0.2μg of the IN-CAT construct was transfected with 44μg of competitor resulting in a 350 molar excess of competitor. Half of the plates were treated with IFN-γ (200U/ml). After 40 hours, cell extracts were prepared and analysed for CAT activity. Transfection of unspecific competitor with the IN-CAT construct reduced its activity sightly (data not shown), but had no influence on its inducibility by IFN-γ; however cotransfection with the IRE prevented the construct from responding to IFN-γ (Fig. 20, left panel). A 250 molar excess of IRE almost completely inhibited the inducibility of the IN-CAT construct by IFN-γ.

In another experiment, 2μg of the same IN-CAT construct (pIN-261/+85CAT) was cotransfected with a 25 molar excess of IRE. This was sufficient to inhibit IFN-γ activation of the IN-CAT construct (Fig. 20, right panel). This is because the lower amount of IFN-γ used in this experiment (50U/ml) was barely sufficient to activate the transcription of the transfected IN-CAT construct (10-fold more was transfected than in the previous experiment) so that a lower amount of

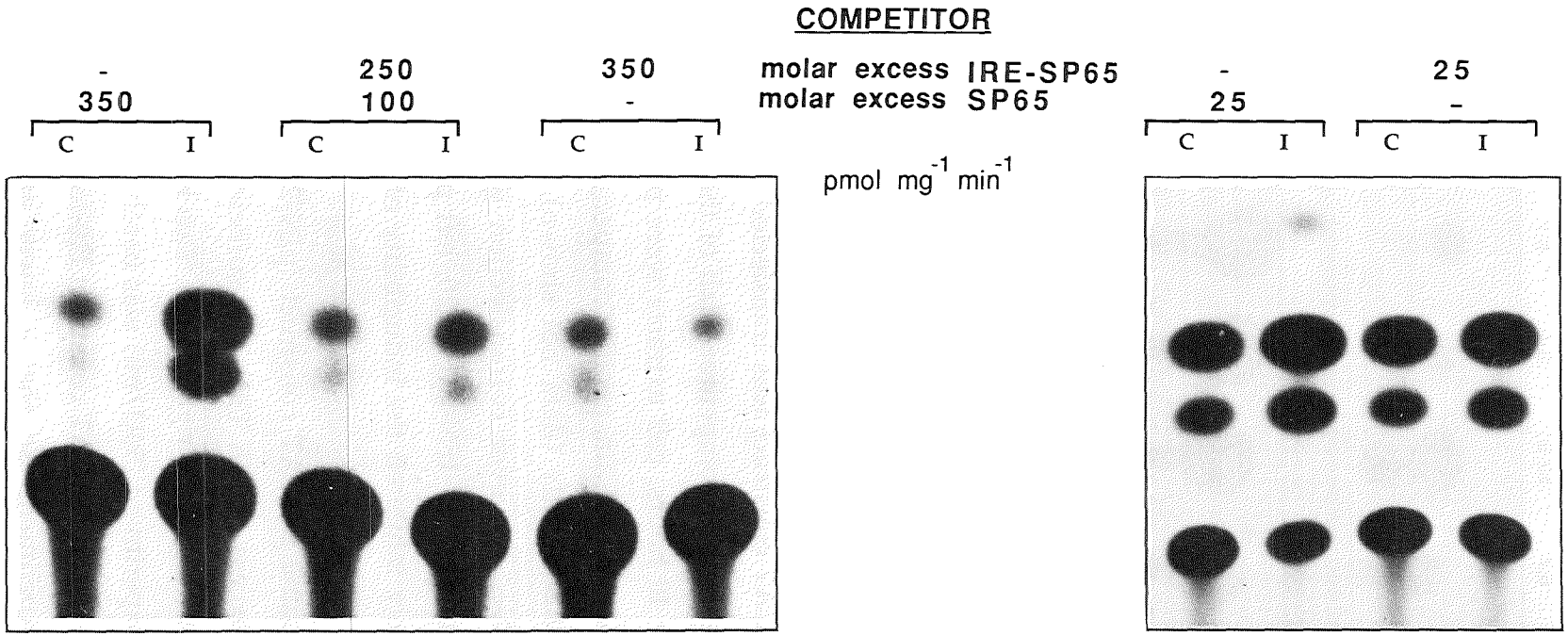


Fig. 20. Competition analysis demonstrates that IFN-γ induces the expression of the murine IN gene through a positively acting regulatory factor.

left panel: 0.2μg pIN-261/+85CAT was cotransfected with 44μg (350 molar excess) of either pSP65 or pIRE-SP65 or with a mixture of both plasmids into GM637 cells by the calcium phosphate method. CAT activity was analysed in the absence (C) or presence (I) of 200U/ml IFN-γ.
right panel: 2μg of pIN-261/+85CAT were cotransfected with 30μg (25 molar excess) of either pSP65 or pIRE-SP65 into GM637 cells. CAT activity was analysed as above except that the IFN-γ concentration was 50U/ml.

competitor was enough to inhibit the IFN-γ activation.
These experiments show that IFN-γ acts positively through the IRE to induce the expression of the murine IN gene.

f. The interferon-γ response element binds an interferon-γ inducible protein complex

I have identified the cis-acting element, the IRE which confers IFN-γ inducibility to the murine IN gene in human fibroblasts and have shown that IFN-γ activates transcription through a positively acting factor. It was of great interest therefore to analyse the proteins which interact with these sequences. This was carried out by an electrophoresis mobility shift assay of a synthetic oligonucleotide corresponding to the IRE after its incubation with extracts from human fibroblast cells. In this assay, the radioactively labelled oligonucleotide was incubated with protein extracts from untreated or IFN-γ treated GM637 cells after which the mix was separated by polyacrylamide gel electrophoresis. The mobility of the oligonucleotide is retarded when proteins bind to it - thus the protein bound DNA is easily distinguished from the unbound DNA, which moves faster through the gel.
The IRE bound three protein complexes (C1, C2 and C3). A slow moving complex (C1) was present in extracts from IFN-γ treated cells only (Fig. 21, lanes 1 and 2). The specificity of the binding of this complex to the IRE was demonstrated by including an excess of unlabelled oligonucleotide in the incubation reaction. A 300 molar excess of unlabelled IRE inhibited the binding of C1 to the IRE (Fig. 21) whereas neither a 300 nor a 500 molar excess of a mutated version of the IRE competed for binding to C1 (Fig. 21). A mutated IRE (mIRE,) containing 14bp alterations, was tested for the binding of proteins under the same conditions as the IRE, but it did not form an IFN-γ induced complex similar to the C1 complex formed with the IRE (Fig. 21, lanes 5 and 6). The complex C3 is unspecific since it is present in extracts from both untreated and IFN-γ treated cells and is not removed by competition. C2 also does not seem bind specifically to the IRE because it does not always bind in every assay.

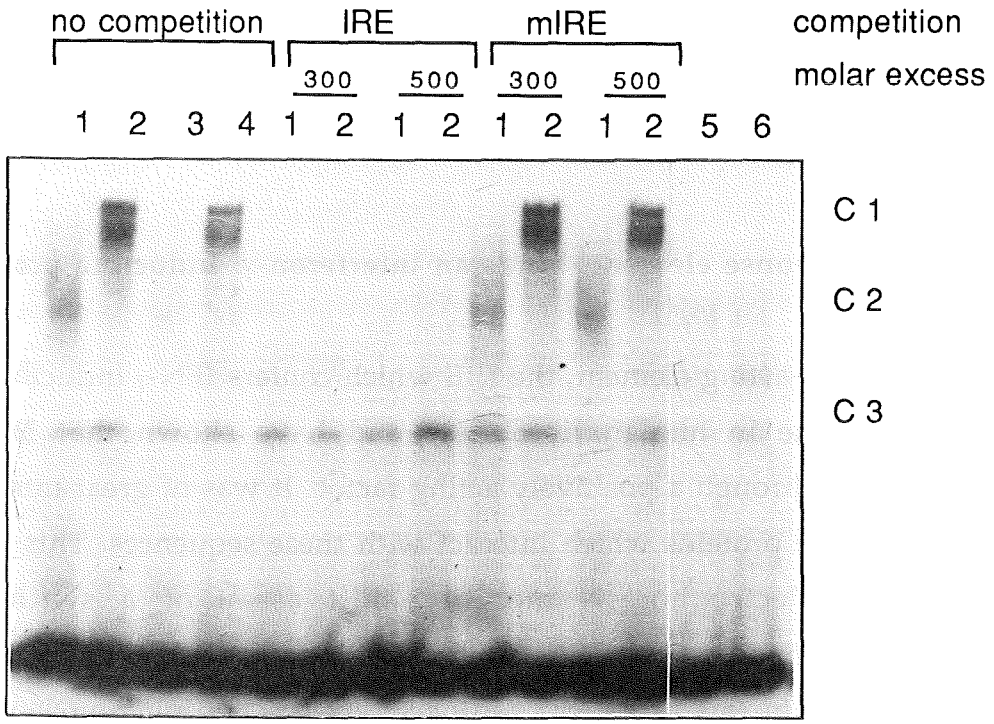


Fig. 21. Analysis of proteins that bind to the IRE of the invariant chain gene.
Lanes 1-4: 4µg nuclear extracts from GM637 cells were incubated with 1fmol end-labelled IRE oligonucleotide corresponding to positions -249 to -221 of the murine IRE. Protein bound DNA was separated from unbound DNA by electrophoresis through a 4% native polyacrylamide gel. C1, C2, C3 are the protein-DNA complexes. 1, extracts from untreated cells; 2, extracts from cells treated with 400U/ml IFN-γ for 24 hours; 3, extracts from cycloheximide treated cells (10µg/ml for 24 hours); 4, extracts from cells treated with 400U/ml IFN-γ and 10µg/ml cycloheximide together for 24 hours.
Competition was achieved by the addition of an excess of unlabelled IRE or a mutated version of the IRE (mIRE; see methods IV.10).
Lanes 5 and 6: 4µg nuclear extracts were incubated with 1fmol end-labelled mIRE oligonucleotide corresponding to a randomly mutated version of the murine IRE (see methods IV.10). 5, extracts from untreated cells; 6, extracts from cells treated with 400U/ml IFN-γ for 24 hours.

C1, the IFN-γ inducible complex is also formed in cells treated with IFN-γ in the presence of cycloheximide, but not in extracts from cells treated with cycloheximide alone (Figs. 21 and 22, lanes 1-4). Thus the formation of this protein complex following IFN-γ treatment does not require protein synthesis, instead, the protein(s) involved are just modified by IFN-γ treatment independently of protein synthesis.

The efficiency of just part of the IRE as competitor was examined, since the IRE comprises two independently defined consensus sequences (see above). Oligonucleotides corresponding to the sequences -247/-238 and -237/-223 were tested as competitor in the protein binding assay. Neither of the oligonucleotides competed for C1 (Fig 22). An unrelated oligonucleotide (-60 element of the human cfos gene) also did not compete (Fig. 22). This means that the IRE (-249/-221) is required to form a stable protein binding complex. Yet these sequences alone are insufficient to confer full IFN-γ inducibility to the IN promoter. This may be explained as follows: The shortest inducible IRE (-254/-215) contains the sequences -249/-221. A protein binding to this region will obviously have several important contacts along its length although it is possible that some binding site(s) that are necessary for transcriptional activation by IFN-γ lie outside the sequences -249/-221.

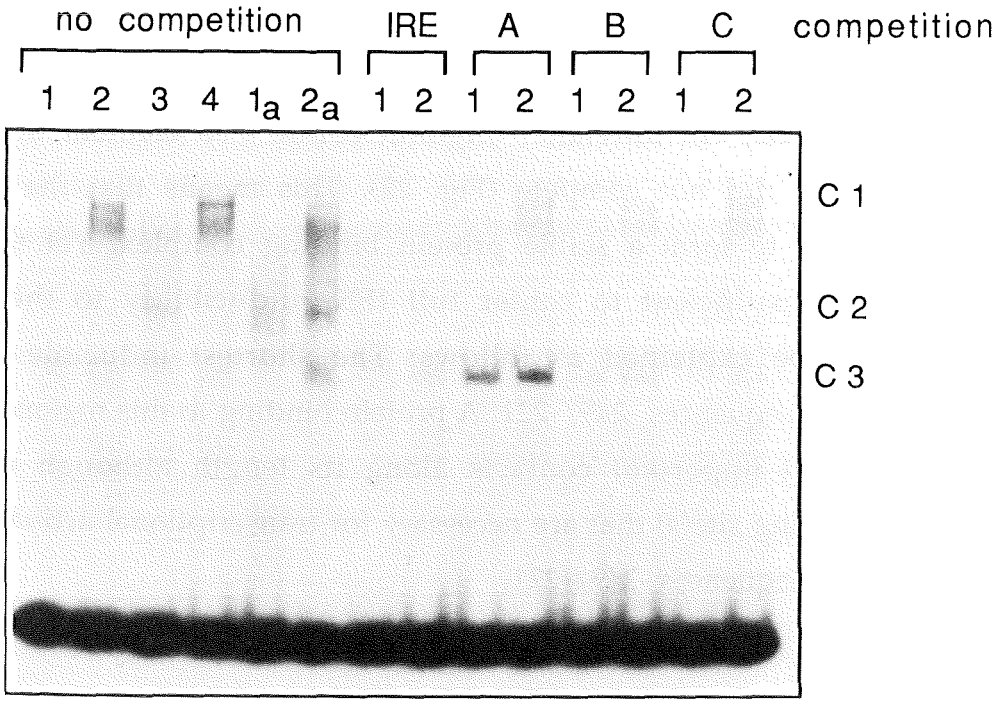


Fig. 22. The binding of the IRE to C1 is not inhibited by parts of the IRE sequence.
4µg nuclear extracts were incubated with 1fmol of end-labelled oligonucleotide corresponding to positions -249 to -221 of the murine IRE. Protein bound DNA was separated from unbound DNA by electrophoresis through a native polyacrylamide gel. C1, C2, C3 are the protein-DNA complexes. 1, extracts from untreated cells; 2, extracts from cells treated with 400U/ml IFN-γ; 3, extracts from cycloheximide treated cells (10µg/ml); 4, extracts from cells treated with IFN-γ and cycloheximide together. 1a and 2a correspond to 1 and 2 except that the extracts were prepared at a different time. This shows that different preparations of cell extracts yield the same results. Competitor A, sequences -247/-238 of the murine IRE (see Table 3); competitor B, sequences -237/-223 of the murine IRE (see Table 3); competitor C, -60 element (-75/-54) of the human cfos gene (M. Büscher, PhD thesis, University of Karlsruhe).

DISCUSSION

Interferon-γ (IFN-γ) induces the expression of the invariant chain (IN) gene in a variety of cell types and not only those of lymphoid origin (Basham and Merigan, 1983; Collins et al., 1984; Rahmsdorf et al., 1986). The chain of events between the binding of IFN-γ to its receptor on the cell surface and the induction of gene expression is still poorly understood. By taking the IN gene as the end point of IFN-γ activation, I have clarified in this work some of the salient features of the signal cascade: I have shown that 1) IFN-γ activates the transcription of the gene 2) this activation requires the synthesis of some intermediate protein(s) 3) an inducible enhancer transmits the signal from IFN-γ to the transcriptional machinary 4) activation through the cis-acting element is mediated by a positively induced factor 5) an IFN-γ inducible factor binds the IRE. Since this investigation involved the characterization of the cis-acting elements required in the IFN-γ regulation, it also yielded the sequence of the 5′ region of the murine IN gene and allowed comparison with the IN human gene.

All of the experiments described were performed with the murine IN gene transfected into human fibroblasts. I showed that the expression of the transfected murine IN gene is induced by human IFN-γ. This demonstrated 1) that the mechanisms governing the expression are conserved between murine and human cells and 2) that the cis-acting sequences required for the inducibility of the murine IN gene by IFN-γ are present within the transfected clone.

Nuclear run-on experiments have demonstrated that IFN-γ induces the transcription of the transfected murine IN gene. Many hormones, in addition to activating transcription of a gene also control steady state levels of its mRNA (Shapiro and Brock, 1985). I demonstrated that IFN-γ does not prolong the half-life of the IN mRNA.

The requirement for protein synthesis in the activation of the transfected IN gene by IFN-γ was demonstrated by including cycloheximide in the analysis (Fig. 3). Thus IFN-γ induces the synthesis of some intermediary protein(s)

which in turn activate the expression of the IN gene. This mechanism of gene activation by IFN-γ would correlate with the observation that receptors or factors encoded by both chromosomes 6 and 21 of the human are required for IFN-γ regulation whereas only those encoded by chromosome 21 are required for IFNs α and β induced gene expression (Jung et al., 1987). The mechanism of activation of the IN by IFN-γ is typical for IFN-γ activation of several genes: IFN-γ induction of expression of the 2′, 5′-oligoadenylate synthetase gene requires protein synthesis but the activation of this gene by IFNs α and β does not (Faltynek et al., 1985). Recently, Giacomini et al. (1988) have shown that IFN-γ induced expression of the genes coding for class II antigens and their associated IN is inhibited by cycloheximide. The same genes are induced to be expressed by interferons α and β in manner independent of protein synthesis.

In contrast, the IFN-γ induction of the expression of the IP-10 gene is not inhibited in the presence of cycloheximide (Luster et al., 1985). The kinetics of expression of this gene following IFN-γ treatment are more rapid than those of the IN gene, it may therefore be regulated differently by IFN-γ. It is possible that IP-10 could be an intermediate in the induction of the IN by IFN-γ in the same way the proto-oncogene, cfos is implicated in the induction of collagenase by other oncogenes and tumour promoters (Schönthal et al., 1988).

Comparison of the sequence of the murine and human invariant chain genes.

Having shown that IFN-γ activates the transcription of the murine IN gene and since DNA sequences that influence transcription are usually located in the 5′ control region of a gene (Serfling et al., 1985), it was necessary to characterize and sequence the 5′ end of the murine IN gene. Primer extension analysis, S1 mapping, and sequencing of IN mRNA and part of the transfected clone a) provided the necessary information required to map the start of transcription within the genomic clone and b) allowed comparison with the human IN gene.

While the two genes are 74% homologous in the region I sequenced, there are two marked differences located in the 5′ transcribed but untranslated regions of the genes:

1) there is only one ATG translation initiator codon in the murine IN gene

whereas there are two in-frame ATGs in the human IN gene. Although translation initiates predominantly at the second ATG initiator codon in the human, where the sequences of the murine and human IN genes are very similar, use of both ATGs results in two of the various forms of the human IN detected in polyacrylamide gel electrophoresis (Strubin et al., 1986a,b). 2) In the murine gene, the 5′ untranslated sequence consists mainly of a repeat element. The triplet "CAG" is repeated 16 times with one base alteration. Whether the element has replaced an ATG initiator codon or whether the human gene has evolved a second ATG initiator codon is not clear. This repeat is also located in many genes in a variety of organisms such as chicken, mouse, rat, drosophila and bovine genes (as identified by sequence comparison with the NIH Genetic Sequence Data Bank). The significance of repeat seqences has long been debated - whether they are present by accident or whether they have some function. For example, brain identifier (ID) elements, a class of middle repetitive sequences, may be involved in the regulation of brain-specific gene expression (Milner et al., 1981). However the presence of the CAG repeat in the murine IN gene is probably accidental since it is not present in the human IN gene and its absence there suggests that it cannot be important in the function of the IN gene.

Heterogeneity was also revealed in the sequence of the IN gene between different murine strains by S1 mapping and primer extension analysis of the 5′ flanking regions of these genes. S1 mapping revealed that the sequence of IN RNA in Balb/c mice is different to that in AKR and C57Bl mice about 50 nucleotides upstream from the Nco I site (Fig. 10) since a protected fragment of 50 nucleotides instead of 88 nucleotides is obtained. Primer extension analysis confirmed the difference between the RNAs but suggested that there is only a very small difference in the length of the 5′ untranslated region (in this analysis, the Balb/c IN sequence is only about 6 nucleotides shorter than the AKR IN sequence). In order to confirm the sequence variation, the Balb/c IN gene would have to be cloned and sequenced.

The IFN-γ response element of the murine IN gene

The murine IN IFN-γ response element (IRE) was defined by deletion analysis

of the 5′ flanking region of the gene cloned in front of the coding region of the indicator gene, chloramphenicol acetyl transferase (CAT). This is the first detailed characterization of an IFN-γ response element.

Analysis of CAT activity of deletion mutants following transient transfection into human fibroblasts in the absence and the presence of IFN-γ revealed that the sequences, -259/-215, are both necessary and sufficient for full activation of gene expression by IFN-γ. The sequences between -254 and -215 are sufficient to confer partial IFN-γ inducibility to the IN gene. The expression of deletion mutants lacking all or just part of these sequences is not inducible by IFN-γ. S1 mapping of stable transfectants of constructs with deletions in the 5′ control region of the murine IN gene confirmed the location of the IRE and extended the analysis of CAT activity by showing that transcription is correctly initiated both in the presence and absence of IFN-γ.

The fact that the IRE functions with the IN promoter cannot exclude the presence of a second element which could be necessary for IFN-γ induction located within the promoter, i. e. between -189 and +85. However, the IRE also functions in front of the tk promoter from the Herpes simplex virus. While this excludes the requirement of another element, it is still possible that a second element which enhances the activation of the IN gene through the IRE, exists between -189 and +85. This point would have to be clarified by testing the IRE in front of various deletion mutants of the IN promoter.

Comparison of the IRE with other interferon inducible genes

A comparison of the DNA sequence of the IRE with other genes that are regulated by IFN-γ or that encode proteins which are associated with the IN gene, revealed three conserved regions of homology - a 9-mer defined by Basta et al. (1987) and a 15-mer and 8-mer defined by O′ Sullivan et al. (1986). The consensus sequences of the homologous regions and the murine IN versions of them are shown in table 3.

While this is the first example showing the functional significance of the 15-mer and 9-mer consensus elements, the 8-mer is not present in the murine IN gene. A sequence very similar to the murine IN IRE is located in the 5′

flanking region of the human IN gene (table 3) but this has not yet been shown to be functionally relevant in IFN-γ induction.

Since the start of this work, several reports on the cis-acting sequences involved in the IFN-γ regulation of genes have appeared in the literature. Boss and Strominger (1986) studied the regulation of a human class II DQβ gene by IFN-γ in fibroblast cells. They found two well conserved sequences in the region required for IFN-γ induction of gene expression (position -159 to the end of the first exon). These correspond to the 15-mer and 8-mer elements of O´ Sullivan et al. (1986). Proteins bind to these conserved regions (Miwa et al., 1987) but it has yet to be demonstrated directly that these elements are involved in IFN-γ regulation.

The expression of the gene IP-10 isolated by Luster et al. (1985) is also induced by IFN-γ. The cis-acting element involved in the regulation of this gene by IFN-γ is different to that I have defined in the murine IN gene (Luster and Ravetch, 1987). Sequences similar to the murine IN IRE are not present in the 5´ flanking region of the IP-10 gene. Instead, the sequences between 250 and 60bp upstream of the transcription initiation site which confer IFN-γ inducibility to the IP-10 gene, are more similar to the IFN α/β consensus sequence defined by Friedman and Stark, (1985). This is not surprising since the kinetics of induction of the IP-10 gene by IFN-γ are more similar to those of IFN α/β induced genes than to those of the murine IN gene (see above) suggesting a different mechanism of regulation of the IP-10 gene by IFN-γ.

The cis-acting element which confers inducibility by IFN-γ to the murine IN gene is very different to that responsible for IFN α and β gene activation of several genes including the 2´, 5´-oligoadenylate synthetase gene (Friedman and Stark, 1985; Levy et al., 1988; Cohen et al., 1988). This was to be expected since the mechanism of gene activation by the type I IFNs and type II IFNs is different (Faltynek et al., 1985).

Mechanism of activation through the IRE

The IRE behaves as an inducible enhancer

The IRE was shown to fulfill the properties of an enhancer (Khoury and Gruss,

1983) because it functions independently of distance from the IN promoter, in an inverted orientation and with a heterologous promoter. It functions both in the presence of IFN-γ to activate transcription and as a basal enhancer, because it increases the level of expression of the tkCAT construct into which it was introduced. Other examples of inducible enhancers include the tumour promoter response element (TRE) of the collagenase gene (Angel et al., 1987) and the estrogen response element (ERE) of the *Xenopus* vitellogenin gene (Klein-Hitpass et al., 1988) and the IFN-α/β response element of the 2′, 5′-oligoadenylate synthetase gene (Cohen et al., 1988; Rutherford et al., 1988).

A positively acting factor binds the IRE

IFN-γ could activate the transcription of the IN gene though the cis-acting IRE in one of two ways: either by the positive induction of a protein to bind the IRE or by the removal of a repressor from the IRE. Cotransfection of a high molar excess of IRE sequences with an IFN-γ inducible construct inhibits the IFN-γ induction of that construct (Fig. 20). This is a direct demonstration that the former possibility is true.

The positively acting protein(s) which bind the IRE were analysed in an electrophoresis mobility shift assay. It was demonstrated that an IFN-γ activated protein complex binds to the IRE (C1, Figs. 21 and 22). This protein bound specifically to the DNA since a high molar excess of unlabelled IRE competed for binding whereas a randomly mutated version of the IRE did not. Neither the 15-mer nor the 9-mer consensus sequences alone competed for binding, indicating the importance of both sequences for the binding of the IFN-γ activated binding factor.

The proteins that bind the IRE are present in cell extracts treated with IFN-γ in the presence of the protein synthesis inhibitor, cycloheximide. This indicates that the proteins seen to bind the IRE are activated by, rather than their synthesis is induced by IFN-γ. This seems to be in contrast to the fact that the induction of expression of the endogenous and the transfected IN genes by IFN-γ is inhibited by cycloheximide, suggesting the involvement of a newly synthesized protein induced by IFN-γ. The reasons for the discrepancy are not

yet clear. Preliminary experiments show that also with minimal promoters which just contain the IRE from positions -259 to -215, the induction by IFN-γ is inhibited by cycloheximide. My experiments show that the sequences which I used for the protein binding studies (-249/-221) transmit only part of the of the IFN response as compared to the complete element (-259/-215). It may be that this somewhat longer sequence binds an additional IFN-γ induced protein which is newly synthesized, or is possible that the minimal IRE from -249 to -221 is indeed capable of binding all the relevant transcription factors *in vivo*, but not *in vitro*, or that the protein in question has been lost during the preparation of the nuclear extracts or that the protein synthesis dependent factor is so small in comparison to the protein synthesis independent factor that it is not detectable in the electrophoresis mobility shift assay. Another possibility is that IFN-γ activates the protein synthesis independent factor to bind to the IRE and a second (protein synthesis dependent) factor to bind somewhere else in the IN promoter to activate IN transcription. Further DNA-protein binding studies and further analysis of the IN promoter should distinguish between these possibilities.

In conclusion, this work demonstrates the mechanism by which IFN-γ induces the expression of the IN gene, defines the cis-acting element which confers IFN-γ inducibility to the gene and demonstrates the binding of a trans-acting factor to this element.

REFERENCES

Adolf, G. R. (1985)

Structure and effects of interferon-gamma. Oncology 42 : 33-40.

Aguet, M. (1980)

High-affinity binding of ^{125}I-labelled mouse interferon to a specific cell surface receptor. Nature. 284 : 459-461.

Aguet, M., Belardelli, F., Blanchard, B., Marcucci, F. and Gresser, I. (1982)

High-affinity binding of ^{125}I-labelled mouse interferon to a specific cell surface receptor.

IV. Mouse γ interferon and cholera toxin do not compete for the common receptor site of α/β interferon. Virol. 117 : 541-544.

Aiyer, R. A., Serrano, L. E. and Jones, P. P. (1986)

Interferon-γ binds to high and low affinity receptor components on murine macrophages. J. Immunol. 136 : 3329-3334.

Angel, P., Baumann, I., Stein, B., Delius, H., Rahmsdorf, H. J. and Herrlich, P. (1987)

12-*O*-tetradecanoyl-phorbol-13-acetate induction of the human colleganase gene is mediated by an inducible enhancer element located in the 5′-flanking region. Mol. Cell. Biol. 7 : 2256-2266.

Auffray, C. and Rougeon, F. (1980)

Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumor RNA. Eur. J. Biochem. 107 : 303-314.

Ausubel, F., Brent, R., Kingston, R., Moore, D., Smith, J., Seidman, J. and Struhl, K. (editors) (1987)

Current protocols in molecular biology. John Wiley and Sons Ltd.

Banerji, J., Rusconi, S. and Schaffner, W. (1981)

Expression of a β-globin gene is enhanced by remote SV40 DNA sequences. Cell 27 : 299-308.

Barberis, A., Superti-Furga, G. and Busslinger, M. (1987)

Mutually exclusive interaction of the CCAAT-binding factor and of a displacement protein with overlapping sequences of a histone gene

promoter. Cell 50 : 347-359.

Basham, T. Y. and Merigan T. C. (1983)

Recombinant interferon-γ increases HLA-DR synthesis and expression. J. Immunol. 130 : 1492- 1494.

Basham, T. Y., Kaminski, M. S., Kitamura, K., Levy, R. and Merigan, T. C. (1986)

Synergistic antitumor effect of interferon and anti-idiotype monoclonal antibody in murine lymphoma. J. Immunol. 137 : 3019-3024.

Basta, P. V., Sherman, P. A. and Ting, J. P.-Y. (1987)

Identification of an interferon-γ response region 5′ of the human histocompatibility leukocyte antigen DRα chain gene which is active in human glioblastoma multiforme lines. J. Immunol. 138 : 1275-1280.

Benacerraf, B. (1981)

Role of MHC gene products in immune regulation. Science 212 : 1229-1238.

Berk, A. J. and Sharp, P. A. (1977)

Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. Cell 12 : 721-732.

Boss, J. M. and Strominger, J. L. (1986)

Regulation of a transfected human class II major histocompatibility complex gene in human fibroblasts. Proc. Natl. Acad. Sci. USA 83 : 9139-9143.

Branca, A. A. and Baglioni, C. (1981)

Evidence that types I and II interferons have different receptors. Nature 294 : 768-770.

Breathnach, R. and Chambon, P. (1981)

Organization and expression of eucaryotic split genes coding for proteins. Ann. Rev. Biochem. 50 : 349-383.

Cato, A. C. B., Henderson, D. and Ponta, H. (1987)

The hormone response element of the mouse mammary tumour virus DNA mediates the progestin and androgen induction of transcription in the proviral long terminal repeat region. EMBO J. 6 : 363-368.

Chandler, V. L., Maler, B. A. and Yamamoto, K. R. (1983)

DNA sequences bound specifically by glucocorticoid receptor in vitro render a heterologous promoter hormone responsive in vivo. Cell 33 : 489-499.

Chen, E. Y. and Seeburg, P. H. (1985)
Supercoil sequencing: a fast and simple method for sequencing plasmid DNA. DNA 4 : 165-170.

Claesson, L. and Peterson, P. A. (1983)
Association of human γ chain with class II transplantation antigens during intracellular transport. Biochemistry 22 : 3206-3213.

Claesson-Welsh, L., Barker, P. E., Larhammer, D., Rask, L., Ruddle, F. H. and Peterson, P. A. (1984)
The gene encoding the human class II antigen-associated gamma chain is located on chromosome 5. Immunogenetics 20 : 89-93

Claesson-Welsh, L. and Peterson, P. A. (1985)
Implications of the invariant γ-chain on the intracellular transport of class II histocompatibility antigens. J. Immunol. 135 : 3551-3557.

Cohen, B., Peretz, D., Vaiman, D., Benech, P. and Chebath, J. (1988)
Enhancer-like interferon responsive sequences of the human and murine (2′-5′) oligoadenylate synthetase gene promoters. EMBO J. 7 : 1411-1419.

Collins, T., Korman, A. J., Wake, C. T., Boss, J. M., Kappes, D. J., Fiers, W., Ault, K. A., Gimbrone, M. A. Jr., Strominger, J. L. and Pober, J. S. (1984)
Immune interferon activates multiple class II major histocompatibility complex genes and the associated invariant chain gene in human endothelial cells and dermal fibroblasts. Proc. Natl. Acad. Sci. USA 81 : 4917-4921.

Cowing, C., Pincus, S. H., Sachs, D. H. and Dickler, H. (1978)
A subpopulation of adherant accessory cells bearing both *I-A* and *I-E* or *C* subregion antigens is required for antigen-specific murine T lymphocyte proliferation. J. Immunol. 121 : 1680-1685.

Day, C. E. and Jones, P. P. (1983)
The gene encoding the Ia antigen-associated invariant chain (Ii) is not linked to the *H-2* complex. Nature 302 : 157-159.

Dignam, J. D., Lebovitz, R. M. and Roeder, R.G. (1983)

Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucl. Acids Res. 11 : 1475-1489.

Dretzen, G., Bellard, M., Sassone-Corsi, P. and Chambon, P. (1981)

A reliable method for the recovery of DNA fragments from agarose and acrylamide gels. J. Biochem. 112 : 295-298.

Dynan, W. S. and Tjian, R. (1983)

The promoter-specific transcription factor Sp1 binds to upstream sequences in the SV40 early promoter. Cell 35 : 79-87.

Faltynek, C. R., McCandless, S., Chebath, J. and Baglioni, C. (1985)

Different mechanisms for activation of gene transcription by interferons α and γ. Virol. 144 : 173-180.

Feinberg, A. P. and Vogelstein, B. (1983)

A technique for radiolabelling DNA restriction endonuclease fragments to a high specific activity. Anal. Biochem, 132 : 6-13.

Friedman, R. L., Manly, S. P., McMahon, M., Kerr, I. M. and Stark, G. R. (1984)

Transcriptional and posttranscriptional regulation of interferon-induced gene expression in human cells. Cell 38 : 745-755.

Friedman, R. L. and Stark, G. R. (1985)

α-Interferon-induced transcription of HLA and metallothionein genes containing homologous upstream sequences. Nature 314 : 637-639.

Giacomini, P., Tecce, R., Gambari, R., Sacchi, A., Fisher, P. B. and Natali, P. G. (1988)

Recombinant human IFN-γ, but not IFN-α or IFN-β, enhances MHC- and non-MHC-encoded glycoproteins by a protein synthesis-dependent mechanism. J. Immunol. 140 : 3073-3081.

Gidoni, D., Dynan, W. S. and Tjian, R. (1984)

Multiple specific contacts between a mammalian transcription factor and its cognate promoters. Nature 312 : 409-413.

Gorman, C. M., Moffat, L. F. and Howard, B. H. (1982)

Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. 2 : 1044-1051.

Graham, F. L. and Van der Eb, A.J. (1973)

A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52 : 456-467.

Gray, P. W., Leung, D. W., Pennica, D., Yelverton, E., Najarian, R., Simonsen, C. C., Derynk, R., Sherwood, P. J., Wallace, D. M., Berger, S. L., Levinson, A. D. and Goeddel, D. V. (1982)

Expression of human immune interferon cDNA in *E. coli* and monkey cells. Nature 295 : 503-508.

Gray, P. W. and Goeddel, D. V. (1987)

Molecular biology of interferon-gamma. Lymphokines 13 : 151-162.

Greiner, G. W., Fisher, P. B., Pestka, S. and Schlom, J. (1986)

Differential effects of recombinant human leukocyte interferons on cell surface antigen expression. Cancer Res. 46 : 4984-4990.

Hamblin, A. S. (1988)

Lymphokines. IRL press limited, Oxford.

Hanahan, D. (1983)

Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. 166 : 557-580.

Hannigan, G. and Williams, B. R. G. (1986)

Transcriptional regulation of interferon-responsive genes is closely linked to interferon receptor occupancy. EMBO J. 5 : 1607-1613.

Holt, G. D., Swiedler, S. J., Freed, J. H. and Hart, G. W. (1985)

Murine Ia-associated invariant chain's processing to complex oligosaccharide forms and its dissociation from the I-A^k complex. J. Immunol. 135 : 399-407.

Hynes, N., Van Ooyen, A. J. J., Kennedy, N., Herrlich, P., Ponta, H., and Groner, B. (1983)

Subfragments of the large terminal repeat cause glucocorticoid responsive expression of mouse mammary tumor virus and of an adjacent gene. Proc. Natl. Acad. Sci. USA 80 : 3637-3641.

Ina, Y., Koide, Y., Nezu, N. and Yoshida, T. O. (1987)

Regulation of HLA class II antigen expression : intracellular signaling

molecules responsible for the regulation by IFN-γ and cross-linking of Fc receptors in HL-60 cells. J. Immunol. 139 : 1711-1717.

Isaacs, A. and Lindenmann, J. (1957)

Virus interference. I. The interferon. Proc. R. Soc. Lond. (Biol.) 147 : 258-267.

Jones, P. P., Murphy, D. B., Hewgill, D. and McDevitt, H. O. (1978)

Detection of a common polypeptide chain in *I-A* and *I-E* sub-region immunoprecipitates. Immunochemistry 16 : 51-60.

Jung, V., Rashidbaigi, A., Jones, C., Tischfield, J. A., Shows, T. B. and Pestka, S. (1987)

Human chromosomes 6 and 21 are required for sensitivity to human interferon γ. Proc. Natl. Acad. Sci. USA 84 : 4151-4155.

Kafatos, F. C., Jones, C. W. and Efstratiadis, A. (1979)

Determination of nucleic acid sequence homologies and relative concentrations by a dot hybridization procedure. Nucl. Acids Res. 7 : 1541-1552.

Kaufmann, J. F., Auffray, C., Korman, A. J., Shackelford, D. A. and Strominger, J. (1984)

The class II molecules of the human and murine major histocompatibility complex. Cell 36 : 1-13.

Kazmaier, M., Brüning, E. and Ryffel, G. U. (1985)

Post-transcriptional regulation of albumin gene expression in *Xenopus* liver. EMBO J. 4 : 1261-1266.

Khoury, G. and Gruss, P. (1983)

Enhancer Elements. Cell. 33 : 313-314.

King, D. P. and Jones, P. P. (1983)

Induction of Ia and H-2 antigens on a macrophage cell line by immune interferon. J. Immunol. 131 : 315-318.

Kirchner, H. (1984)

Interferons, a group of multiple lymphokines. Springer Semin. Immunopathol. 7 : 347-374.

Klein-Hitpass, L., Ryffel, G. U., Heitlinger, E. and Cato, A. C. B. (1988)

A 13 bp palindrome is a functional estrogen responsive element and interacts specifically with estrogen receptor. Nucl. Acids Res. 16 : 647-663.

Koch, N., Koch, S. and Hämmerling, G. J. (1982)

Ia invariant chain detected on lymphocyte surfaces by monoclonal antibody. Nature 299 : 644-645.

Koch, N., Wong, G. H. W. and Schrader, J. W. (1984)

Ia antigens and associated invariant chain are induced simultaneously in lines of T-dependent mast cells by recombinant interferon-γ. J. Immunol. 132 : 1361-1369.

Koch, N., Lauer, W., Habicht, J. and Dobberstein B. (1987)

Primary structure of the gene for the murine Ia antigen-associated invariant chains (Ii). An alternatively spliced exon encodes a cysteine-rich domain highly homologous to a repetitive sequence of thyroglobulin. EMBO J. 6 : 1677-1683.

Koch, S., Zalcberg, J. R. and McKenzie, I. F. C. (1984)

Description of a murine B lymphoma tumor-specific antigen. J. Immunol. 133 : 1070-1077.

Kudo, J., Chao, L.-Y., Narni, F. and Saunders, G. F. (1985)

Stucture of the human gene encoding the invariant γ-chain of class II histocompatibility antigens. Nucl. Acids Res. 13 : 8827-8841.

Kusari, J. and Sen, G. C. (1987)

Transcriptional analyses of interferon-inducible mRNAs. Mol. Cell. Biol. 7 : 528-531.

Landolfo, S., Arnold, B. and Suzan, M. (1982)

Immune (γ) interferon production by murine T cell lymphomas. J. Immunol. 128 : 2807-2809.

Lengyel, P. (1982)

Biochemistry of interferons and their actions. Ann. Rev. Biochem. 51 : 251-282.

Levy, D. E., Kessler, D. S., Pine, R., Reich, N. and Darnell, J. E. Jr. (1988)

Interferon-induced nuclear factors that bind a shared promoter element correlate with positive and negative transcriptional control. Genes and

Development 2 : 383-393.

Lipp, J. and Dobberstein, B. (1986)

Signal recognition particle-dependent membrane insertion of mouse invariant chain: a membrane-spanning protein with a cytoplasmically exposed amino terminus. J. Cell Biol. 102 : 2169-2175.

Littman, S. J., Faltynek, C. R. and Baglioni, C. (1985)

Binding of human recombinant ^{125}I-interferon γ to receptors on human cells. J. Biol. Chem. 260 : 1191-1195.

Lowry, O., Rosebrough, N., Farr, A., Randall, R., (1951)

Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193 : 265-275.

Luckow, B. and Schütz, G. (1987)

CAT constructions with multiple unique restriction sites for the functional analysis of eukaryotic promoters and regulatory elements. Nucl. Acids Res. 15 : 5490.

Luster, A. D., Unkeless, J. C. and Ravetch, J. V. (1985)

γ-Interferon transcriptionally regulates an early-response gene containing homology to platelet proteins. Nature 315 : 672-676.

Luster, A. D. and Ravetch, J. V. (1987)

Genomic characterization of a gamma-interferon-inducible gene (IP-10) and identification of an interferon-inducible hypersensitive site. Mol. Cell. Biol. 7 : 3723-3731.

Lynes, M. A., Lanier, L. L., Babcock, G. F., Wettstein, P. J. and Haughton, G. (1978)

Antigen-induced murine B cell lymphomas. I. Induction and characterization of CH1 and CH2. J. Immunol. 121 : 2352-2357.

Machamer, C. E. and Cresswell, P. (1982)

Biosynthesis and glycosylation of the invariant chain associated with HLA-DR antigens. J. Immunol. 129 : 2564-2569.

Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982)

Molecular cloning. A laboratory manual. Cold Spring Harbour Laboratory, N.Y.

Marks, M. S. and Cresswell, P. (1986)

Invariant chain associates with HLA class II antigens via its extracytoplasmic region. J. Immunol. 136 : 2519-2525.

Maxam, A. M. and Gilbert, W. (1980)

Sequencing end-labeled DNA with base-specific chemical cleavages. Methods in Enzymology 65 : 499-560

McKnight, S. L. and Kingsbury, R. (1982)

Transcriptional control signals of a eukaryotic protein-coding gene. Science 217 : 316-324.

McMaster, G. K. and Carmichael, G. G. (1977)

Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. Proc. Natl. Acad. Sci. USA 74 : 4835-4838.

Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K. and Green, M. R. (1984)

Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucl. Acids Res. 12 : 7035-7056

Miller, J. and Germain, R. N. (1986)

Efficient cell surface expression of class II MHC molecules in the absence of associated invariant chain. J. Exp. Med. 164 : 1478-1489.

Milner, R. J., Bloom, F. E., Lai, C., Lerner, R. A. and Sutcliffe, J. G. (1984)

Brain-specific genes have identifier sequences in their introns. Proc. Natl. Acad. Sci. USA 81 : 713-717.

Minty, A. J., Caravatti, M., Robert, B., Cohen, A., Daubas, P., Weydert, A., Gros, F. and Buckingham, M. E. (1981)

Mouse actin messenger RNAs. Construction and characterization of a recombinant plasmid molecule containing a complimentary DNA transcript of mouse α-actin mRNA. J. Biol. Chem. 256 : 1008-1014.

Miwa, K., Doyle, C. and Strominger, J. L. (1987)

Sequence-specific interactions of nuclear factors with conserved sequences of human class II major histocompatibility complex genes. Proc. Natl. Acad. Sci. USA 84 : 4939-4943.

Nakamura, M., Manser, T., Pearson, G. D. N., Daley, M. J. and Gefter, M. L. (1984)
Effect of IFN-γ on the immune response *in vivo* and on gene expression *in vitro*. Nature 307 : 381-382.

O'Sullivan, D. M., Larhammar, D., Wilson, M. C., Peterson, P. A. and Quaranta, V. (1986)
Structure of the human Ia-associated invariant (γ)-chain gene: identification of 5' sequences shared with major histocompatibility complex class II genes. Proc. Natl. Acad. Sci. USA 83 : 4484-4488.

O'Sullivan, D. M., Noonan, D. and Quaranta, V. (1987)
Four Ia invariant chain forms derive from a single gene by alternate splicing and alternate initiation of transcription/translation. J. Exp. Med. 166 : 444-460.

Paulnock-King, D., Sizer, K. C., Freund, Y. R., Jones, P. P. and Parnes, J. R. (1985)
Coordinate induction of Ia α, β, and I_i mRNA in a macrophage cell line. J. Immunol. 135 : 632-636.

Pestka, S., Langer, J. A., Zoon, K. C. and Samuel, C. E. (1987)
Interferons and their actions. Ann. Rev. Biochem. 56 : 727-777.

Ponta, H., Kennedy, N., Skroch, P., Hynes, N. E. and Groner, B. (1985)
Hormonal response region in the mouse mammary tumor virus long terminal repeat can be dissociated from the proviral promoter and has enhancer properties. Proc. Natl. Acad. Sci. USA 82 : 1020-1024.

Porter, A. C. G., Chernajovsky, Y., Dale, T. C., Gilbert, C. S., Stark, G. R. and Kerr, I. M. (1988)
Interferon response element of the human gene 6-16. EMBO J. 7 : 85-92.

de Préval, C., Lisowska-Grospierre, B., Loche, M., Griscelli, C. and Mach, B. (1985)
A *trans*-acting class II regulatory gene unlinked to the MHC controls expression of HLA class II genes. Nature 318 : 291-293.

Ptashne, M. (1986)
Gene regulation by proteins acting nearby and at a distance. Nature 322 : 697-698.

Rabourdin-Combe, C. and Mach, B. (1983)
Expression of HLA-DR antigens at the surface of mouse L cells

co-transfected with cloned human genes. Nature 303 : 670-674

Rahmsdorf, H. J., Koch, N., Mallick, U. and Herrlich, P. (1983)

Regulation of MHC class II invariant chain expression: induction of synthesis in human and murine plasmocytoma cells by arresting replication. EMBO J. 2 : 811-816.

Rahmsdorf, H. J., Forni, L., Mallick, U. and Herrlich, P. (1984)

Regulation of MHC class II-associated invariant chain synthesis. Disease Markers 2 : 161-168.

Rahmsdorf, H. J., Harth, N., Eades, A.-M., Litfin, M., Steinmetz, M., Forni, L. and Herrlich, P. (1986)

Interferon-γ, mitomycin C, and cycloheximide as regulatory agents of MHC class II-associated invariant chain expression. J. Immunol. 136 : 2293-2299.

Rutherford, M. N., Hannigan, G. E. and Williams, B. R. G. (1988)

Interferon-induced binding of nuclear factors to promoter elements of the 2-5A synthetase gene. EMBO. J. 7 : 751-759.

Sanger, F., Nicklen, S. and Coulson, A. R. (1977)

DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74 : 5463-5467.

Sant, A. J., Cullen, S. E., Schwartz, B. D. (1985)

Biosynthetic relationships of the chondroitin sulfate proteoglycan with Ia and invariant chain glycoproteins. J. Immunol. 135 : 416-422.

Schneider, F.-J., Opel, B., Ballhausen, W., Henkes, W., Steinlein, P. and Reske, K. (1987)

Synthesis and expression of MHC class II molecules in the absence of attached invariant chains by recombinant interferon-γ-activated bone marrow-derived macrophages. Eur. J. Immunol. 17 : 1235-1242.

Schöler, H. R. and Gruss, P. (1985)

Cell type-specific transcriptional enhancement *in vitro* requires the presence of *trans*-acting factors. EMBO J. 4 : 3005-3013.

Schönthal, A., Herrlich, P., Rahmsdorf, H. J. and Ponta, H. (1988)

Requirement for *fos* gene expression in the transcriptional activation of collagenase by other oncogenes and phorbol esters. Cell 54 : 325-334.

Sekaly, R. P., Tonnelle, C., Strubin, M., Mach, B. and Long, E. O. (1986)
Cell surface expression of class II histocompatibility antigens occurs in the absence of the invariant chain. J. Exp. Med. 164 : 1490-1504.

Sekaly, R. P., Jacobson, S., Richert, J. R., Tonnelle, C., McFarland, H. F. and Long, E. O. (1988)
Antigen presentation to HLA class II-restricted measles virus-specific T-cell clones can occur in the absence of the invariant chain. Proc. Natl. Acad. Sci. USA 85 : 1209-1212.

Serfling, E., Jasin, M. and Schaffner, W. (1985)
Enhancers and eukaryotic gene transcription. Trends in Genetics 1 : 224-230.

Shapiro, D. J. and Brock, M. L. (1985)
Messenger RNA stabilization and gene transcription in the estrogen induction of vitellogenin mRNA. Biochemical Actions of Hormones 12 : 139-172.

Singer, P. A., Lauer, W., Dembić, Z., Mayer, W. E., Lipp, J., Koch, N., Hämmerling, G., Klein, J. and Dobberstein, B. (1984)
Stucture of the murine Ia-associated invariant (Ii) chain as deduced from a cDNA clone. EMBO J. 3 : 873-877.

Southern, E. M. (1975)
Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98 : 503-517.

Southern, P. J. and Berg, P. (1982)
Transformation of mammalian cells to antibiotic resistance with a bacterial gene under the control of the SV40 early region promoter. J. Mol. Appl. Genetics 1 : 327-341.

Spandidos, D. A. and Wilkie, N. M. (1984)
Expression of exogenous DNA in mammalian cells. In : Transcription and translation - a practical approach. (Eds.) B. D. Hames and S. J. Higgans. IRL Press Oxford and Washington D. C.

Spindler, S. R., Mellon, S. H. and Baxter, J. D. (1982)
Growth hormone gene transcription is regulated by thyroid and

glucocorticoid hormones in cultured rat pituitary tumor cells. J. Biol. Chem. 19 : 11627-11632.

Steinmetz, M., Minard, K., Horvath, S., McNicholas, J., Srelinger, J., Wake, C., Long, E., Mach, B. and Hood, L. (1982)
A molecular map of the immune response region from the major histocompatibility complex of the mouse. Nature 300 : 35-42.

Stockinger, B., Pessara, U., Lin, R. H., Habicht, J., Grez, M. and Koch, N. (1988)
A role of Ia-associated invariant chains in antigen processing and presentation. Cell, in press.

Stone-Wolff, D. S., Yip, Y. K., Kelker, H. C., Le, J., Henriksen-Destefano, D., Rubin, B. Y., Rinderknecht, E., Aggarwal, B. B. and Vilcek, J. (1984)
Interrelationships of human interferon-gamma with lymphotoxin and monocyte cytotoxin. J. Exp. Med. 159 : 828-843.

Storch, E. and Kirchner, H. (1982)
Induction of interferon in murine bone marrow-derived macrophage cultures by 10-carboxymethyl-9-acridine. Eur. J. Immunol. 12 : 793-796.

Strubin, M., Long, E. O. and Mach, B. (1986a)
Two forms of the Ia antigen-associated invariant chain result from alternative initiations at two in-phase AUGs. Cell 47 : 619-625.

Strubin, M., Berte, C. and Mach, B. (1986b)
Alternative splicing and alternative initiation of translation explain the four forms of the Ia antigen-associated invariant chain. EMBO J. 5 : 3483-3488.

Sugita, K., Miyazaki, J.-I., Appella, E. and Ozato, K. (1987)
Interferons increase transcription of a major histocompatibility class I gene via a 5′ interferon consensus sequence. Mol. Cell. Biol. 7 : 2625-2630.

Takiguchi, M., Ting, J. P. -Y., Buessow, S. C., Boyer, C., Gillespie, Y. and Frelinger, J. A. (1985)
Response of glioma cells to interferon-gamma: increase in class II RNA, protein and mixed lymphocyte reaction-stimulating ability. Eur. J. Immunol. 15 : 809-814.

Vieira, J. and Messing, J. (1982)

The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19 : 259-268.

Weil, J., Epstein, C. J., Epstein, L. B., Sedmak, J. J., Sabran, J. L. and Grossberg, S. E. (1983)

A unique set of polypeptides is induced by γ interferon in addition to those induced in common with α and β interferons. Nature 301 : 437-439.

Wharton, K. A., Yedvobnick, B., Finnerty, V. G. and Artavanis-Tsakonas, S. (1985)

opa : A novel family of transcribed repeats shared by the *notch* locus and other developmentally regulated loci in *D. melanogaster*. Cell 40 : 55-62.

Wheelock, E. F. (1965)

Interferon-like virus-inhibitor induced in human leukocytes by phytohemagglutinin. Science 149 : 310-311.

Wong, G. H. W., Clark-Lewis, I., McKimm-Breschkin, J. L., Harris, A. W. and Schrader, J. W. (1983)

Interferon-γ induces enhanced expression of Ia and H-2 antigens on B lymphoid, macrophage, and myeloid cell lines. J. Immunol. 131 : 788-793.

Yamamoto, K., Floyd-Smith, G., Franke, U., Koch, N., Lauer, W., Dobberstein, B. and Hämmerling, G. J. (1985a)

The gene coding for the Ia-associated invariant chain is located on chromosome 18. Immunogenetics 21 : 83-90.

Yamamoto, K., Koch, N., Steinmetz, M. and Hämmerling, G. J. (1985b)

One gene encodes two distinct Ia-associated invariant chains. J. Immunol. 134 : 3461-3467.

Zecher, R., Ballhausen, W., Reske, K., Linder, D., Schlüter, M. and Stirm, S. (1984)

The invariant chains of mouse class II antigens: biochemical properties and molecular relationship. Eur. J. Immunol. 14 ; 511-517.

Zullo, J. N., Cochran, B. H., Huanf, A. S. and Stiles, C. D. (1985)

Platelet-derived growth factor and double-stranded ribonucleic acids stimulate expression of the same genes in 3T3 cells. Cell 43 : 793-800.